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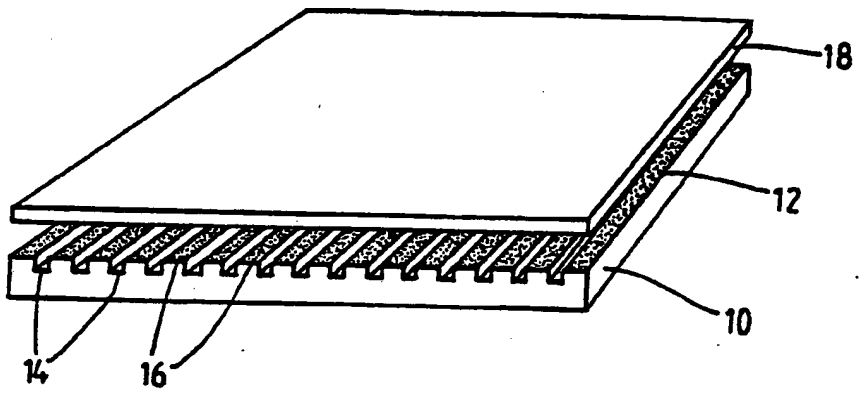

(57) Abstract

A method of electrochemically patterning a surface comprises providing an electrolyte overlying the surface and an array of electrodes adjacent the surface and in contact with the electrolyte, and altering the potential of one or more electrodes of the array so as to deposit or remove or chemically modify a substance on the surface adjacent the electrode. Several such treatments can be performed in sequence, using different electrodes of the array. The method is particularly suitable for step-wise chemical synthesis e.g. of oligonucleotides or other oligomers tethered to the surface. Electrode arrays for use in the method are also claimed.

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ELECTROCHEMICAL TREATMENT OF SURFACES

Introduction and Summary of Invention

The ability to make devices with patterns of specific materials printed on the surface has transformed the manufacture of electronic and electrical components, and the discovery of new materials such as semiconductors has made possible devices for a wide range of applications. The properties of a device depend on the nature and the pattern of the materials on the surface and much research goes into devising new materials and new methods of fabrication, in order to improve production of existing devices and to develop devices with novel applications.

In one aspect, the invention provides a method of electrochemically treating a region of a surface, which method comprises providing an electrolyte overlying the surface and an electrode adjacent the region of the surface to be treated and in contact with the electrolyte, and altering the potential of the electrode so as to deposit or remove or chemically modify a substance on the surface at the region adjacent the electrode.

In another aspect the invention provides an array of electrodes, suitable for use in the stated method, comprising a block of insulating material having a surface, and deposits of electrically conducting material spaced apart in an array on the surface, each deposit being provided with electrical connecting means for altering its potential. Preferably the deposits of electrically conducting material are in the form of parallel-lines spaced apart

by no more than 0.5 mm.

Preferably an array of electrodes is used to treat several regions of the surface simultaneously, and one or more of the electrodes of the array may be used as counter-electrodes. For the purpose of performing several electrochemical treatments in sequence, the electrodes of the array are preferably connected up so that each treatment is performed by altering the potential of a chosen set of one or more of the electrodes of the array.

Altering the potential of an electrode generally results in the generation of a reagent at the surface of the electrode. This reagent may itself be deposited on the surface. Or it may react with some other species, in the electrolyte or on the surface, so as to deposit or chemically modify a substance on the surface. For example, altering the potential of an electrode may generate an acid which removes an acid labile protecting group from a substance on the surface.

The method described in this specification thus provides a convenient alternative to existing methods for modifying surfaces in local areas. It uses the chemical reactions which occur at the surface of an electrode immersed in a solution of electrolyte. Substances which come into contact with the anode or cathode may be modified by electrochemical reactions. These reactions have been studied over many decades and are now well understood. In the most direct application to patterning a surface the electrode would be placed in direct contact with the surface to be modified and current applied; molecules in the surface which contact the electrode would be subject to electrochemical reaction. Alternatively, the radicals and ions generated in the immediate vicinity of an electrode, either those generated by primary electrolysis, or secondary products generated by

interaction of the primary products with the solvent or solutes, may react with an adjacent surface. The location, size and shape of the area that is modified on the surface are determined by the dimensions and position of the electrodes. By contrast with existing methods which use electrochemical processes for electrodeposition or etching, it should be noted that the present method does not require that the surface to be modified forms one of the electrodes, so that the method can be applied to non-conducting materials.

Advantages of the Invention

Electrochemical patterning has a range of applications and potential for automation which give it advantages over many existing methods. The most commonly used method for making small devices is photolithography. In this method, the surface is first coated with a light-sensitive resist, exposed through a mask and the pattern is revealed by etching away the exposed or the unexposed resist and, subsequently, a surface layer. A separate mask must be made for each pattern. There are problems in controlling the etching reaction and in registering masks between each step. Electrolytic patterning can be carried out using a permanent array of independently switched electrodes. An unlimited range of patterns can be created from a dense array of point electrodes, and as will be shown, many complex patterns can be made from a simple array of linear electrodes. Methods for the fabrication of such arrays and circuitry to control the switching are already available. Different shapes can be produced from the same array at different stages in the fabrication by simply switching a different pattern of electrodes without the need to move the array, eliminating the problem of registration. Different effects can be achieved by altering the composition of

the electrolyte, the potential applied to the electrodes, and the duration of the electrolytic pulse, giving the method a versatility not found in alternatives. It would also allow for reproducible production of many identical components from the same tool.

Processes for making small devices must be able to render fine detail. The electrolytic method has high resolution and definition; diffusion of the electrode products is limited by barriers created by the electric field, so that the action of the agents is confined to the region of the surface which is directly opposite the electrode which generates them. The resolution is limited by the size of the electrodes. Current technology could make individual electrodes of sub-micron size, arranged in arrays with electrodes at spacings of a few microns.

The range of reagents that can be produced electrochemically includes radicals, radical ions, acids and bases of any strength. Thus a wide range of chemical modifications can be envisaged; for example oxidations and reductions, acid and base catalysed reactions, polymer formation, chemical etching etc. The method may therefore open up possibilities which are difficult or impossible using existing technology. In particular it may be useful for the fabrication of devices carrying arrays of complex chemical substances made by stepwise synthesis, for example chemical sensors; and for the fabrication of hybrid devices, for example, solid state devices which combine on a single surface, sensor molecules and circuitry to measure interactions between sensors and ligands. The fine regulation that can be applied to the electrolytic current provides a degree of control over reactions which is difficult to achieve using conventional reagents and will be an aid to complex fabrication.

As compared with alternatives, the method is simple and versatile, and lends itself readily to automation.

More detailed consideration of some features of the method

1. Controlling the extent of reaction.

The amount of a reagent that is generated at the surface to be modified can be controlled in several different ways. In the examples described below it was regulated by altering the voltage applied to the electrodes. The concentration of protons generated at the anode depended on the anode current; the extent of reaction depended on this concentration and the time for which the ions were kept in contact with the surface. An alternative is to apply voltage to the electrodes until the component ions of the electrolyte have completely separated; in this case, the concentration of reagent generated at the electrode depends on the initial concentration of ions and the total amount of reagent per unit area depends on the depth of liquid between the electrode and the surface to be modified. The extent of reaction on the surface can be limited by the concentration and thickness of the film of electrolyte.

The most powerful way of regulating the extent of reaction exploits the circuitry which is a necessary component of the system. The electrolytic process and the changes to the reactive surface modify the electrical properties of the system. These changes can be monitored and the information used to modulate the voltage or to switch off the current at the end point.

2. Confining the area affected by electrical caging of ions

If significant time is needed for the reagent

to interact with the surface, it is desirable to prevent the reagent from diffusing away. Ions can be held in place by applying a low potential, sufficient to counteract diffusion but not enough to generate significant amounts of new reagent. This principle has been tested by generating acid at anodes using the method described in the second example. The electrode array was placed against a strip of pH indicator paper. Short pulses of high potential produced faint lines of acid response; long periods of low potential produced no acid response; a short pulse of high potential followed by a longer period at low potential produced a strong acid response in a sharp line; a longer pulse of high potential produced a broader band of acid.

3. Controlling side reactions

Electrolysis often produces a discharge of gases at the electrodes and clearly the release of bubbles could present problems as they would stir the reagents away from the gap between the electrode and the reaction site. However, in the physical embodiment envisaged the amount of gas is small because the film of electrolyte is only a few micrometres thick. In the experiment described in the second example below, there was no visible formation of bubbles during the short pulses of applied potential or during the period when the ions were caged at low potential. Any gas that did come out of solution must have formed microscopically small bubbles under the conditions used.

Other undesirable side reactions could include the formation of radicals in reactions where acids or bases are called for. These may be removed by including radical scavengers. Any undesirable generation of acid or base, which may occur for example when the desired reagent is an oxidising or reducing agent, can be prevented by choosing an electrolyte which produces weak acid or base, such as those used in

pH buffers.

Reference is directed to the accompanying drawings in which:-

Figure 1 is a diagram showing a protocol for building an array of all 256 tetranucleotides.

Figure 2a) and b) show an array of electrodes for use in this invention.

Figure 2c) is an enlargement of the region circled in 2b), showing ions generated at the anodes and cathodes.

Figure 2d) is a further enlargement showing acid deprotection induced in contact with the anode.

Figure 3 is a graph of radioactive counts against Pixel position (88 µm spacing).

Example applications: Novel devices carrying complex patterns of chemicals

It is envisaged that electrolytic patterning will find many and diverse applications. As an illustration of the potential in one field, the following section describes a generic set of applications - the fabrication of small devices with many different chemical compounds tethered to the surface. Such devices have potential in chemical and biochemical analyses which measure interactions between specific ligands and test substances. Examples are enzymes with their substrates, antibodies with antigens, drugs with their target receptors, and nucleic acids with oligonucleotides.

For many applications, it is desirable to compare the interactions of large numbers of ligands of related structure, for example peptides or oligonucleotides of different sequence, or drugs with different modifications to a basic structure. Large numbers of analyses are time consuming if carried out one at a time, and moreover, it is difficult to compare

reactions done at different times as conditions may vary. These problems are removed if multiple ligands are synthesised on a single surface as they can then be reacted simultaneously with the test substance and analysed together. We have demonstrated the power of this approach by synthesising thousands of oligonucleotides on the surface of a glass plate, hybridising radioactive nucleic acids to them, and analysing the pattern of interactions by autoradiography or phosphorimaging. Such large numbers of analyses, which would take many months of work using conventional methods, can be carried out in a day using a matrix of ligands and parallel processing, and have allowed us to carry out wide ranging analyses of the interactions between oligonucleotides (Maskos and Southern, 1992a and 1992b).

At the present time there is a need for methods of nucleic acid sequence analysis which can be automated so that they can be applied on a large scale (Hunkapiller et al., 1991). Devices carrying complete sets of all sequences oligonucleotides of a given length can be used in sequence analysis. If a nucleic acid molecule is hybridised to a such a set, it is possible in principle to determine its sequence, by overlapping those oligonucleotides which give a positive signal. Several authors have considered the theoretical basis of this process (Drmanac and Crkvenjakov, 1987; Bains and Smith, 1988; Lysov et al. 1988; Southern, 1988; Drmanac et al. 1989; Khrapko et al., 1989; Bains, 1991). The length of sequence that can be analysed by a complete set of oligonucleotides of a given length is approximately the square root of the number of oligonucleotides in the set. Octanucleotides, of which there are 65, 536, may be useful in the range up to 200 bases (Lysov et al., 1988; Khrapko et al., 1989; Pevzner, 1989; Maskos,

1991); and decanucleotides, of which there are more than a million, may analyse up to a kilobase. Despite intensive effort it has proved difficult to fabricate arrays with such large numbers of oligonucleotides.

A method has been developed for synthesising, oligonucleotides in situ using a linker which leaves them covalently attached to the surface of a glass plate and available for hybridisation (Maskos, 1991; Southern and Maskos, 1988), and so the problem to be solved is one of carrying out syntheses in small confined areas. The synthesis of an oligonucleotide is a cyclic process consisting of a coupling step in which a monomer unit for one of the four bases is added to the growing chain. A protecting group is then removed from the 5´-hydroxyl group which becomes available for the addition of the next monomer unit. Different sequences can be made in different regions of a surface by confining the reagent during the coupling step or during deprotection. One approach that has been suggested is to print masks over the surface with patterns specific to each coupling or deprotection step (Southern, 1988). A second starts by making small patches of activated polyacrylamide gel on a glass surface; presynthesised oligonucleotides are attached by applying them to the gel by micromanipulation (Khrapko et al., 1989 and 1991); patches 30 x 30 µm can be made in this way but it is an extremely slow and difficult process and it is unlikely that large numbers could be made in this way. A third has been to use photolabile protecting groups which are subsequently removed by illuminating the surface through a patterned mask (Fodor et al., 1991) patches ca. 50 x 50 µm can be treated, but the protecting groups are not very labile and it has not proved possible to make oligonucleotides longer than trimers by this method, though it has been more successful in making

oligopeptides. The largest arrays that have been made comprise 4096 oligonucleotides on a surface 200 x 200 mm (Maskos, 1991). These were made using templates clamped against the surface to form channels through which reagents were passed to irrigate the surface in lines 3 mm wide. Arrays representing all sequences can be built by this procedure using a protocol which applies nucleotide precursors to the surface of the plate in rows and columns; the logic of the procedure is similar to the familiar way of writing the triplet code in which all 64 triplets can be represented just once in 16 rows and four columns. A protocol that could be used to build an array of all 256 tetranucleotides is shown in Fig. 1. This process can be continued to any chosen depth, to produce a two dimensional array of all oligonucleotides of any length in which each oligonucleotide sequence occurs only once. If all four bases are used, 4^s oligonucleotides of length s are synthesised in s steps, applying the nucleotide precursors in $\sqrt{4^s}/2$ rows and columns. Other shapes than stripes can be used to make complete sets of oligomers. The same effect could be achieved by nesting quadrants inside squares that decreased in area fourfold at each step.

Referring to Figure 1, the sequences of four bases in each of the 256 boxes represent a complete set of tetranucleotides. These can be made by applying the base specific precursors in rows and columns. To make this set by the irrigation method, the four bases are first applied in narrow channels in the order A,C,G,T,A,C.... as indicated. After deprotection of the 5'-hydroxyl by treating the whole plate with acid, the channels are turned through 90° and the precursors applied in the same order. The process is continued in the third and fourth cycles but now the width of the channels is increased to embrace four of the narrower

channels.

The protocol can be adapted for electrolytic processing. The device would comprise a set of 16 Pt strips, each switchable between an anode or a cathode. The array would be placed against the derivatised plate and anode current applied to the 'A' channels. this would deprotect the linker in the 1st, 5th, 9th and 13th columns. The electrolyte would be replaced by the precursor for A. After coupling, electrolyte would be introduced and lines 2, 6, 10 and 14 switched to anodes, followed by coupling with C. These would be followed by similar steps for coupling G and T to their columns. A second cycle with the electrodes turned through 90° would produce an array comprising 16 copies of all 16 dinucleotides. The third and fourth bases would be added by a similar process in which A was coupled in the first four columns and rows, C in the second group of four, and so on, to produce an array of all 256 tetranucleotides. Larger sets of longer oligonucleotides can be produced by expanding the groups of lines by a factor of four at each cycle.

A device carrying all octanucleotides made by the existing irrigation procedure would be an unwieldy 750 x 750 mm. Electrolytic deprotection can produce much smaller cells, as illustrated by the following experiment.

Example 1

A standard glass microscope slide was derivatised with an aliphatic linker bearing a primary hydroxyl group at the end of a 20 atom aliphatic chain. A 5'-dimethoxytritylthymidine-H-phosphonate was attached to this hydroxyl by a standard synthetic step (Froehler et al., 1986), over the whole surface of the slide. The slide was immersed in a solution of triethylammonium sulphate in acetonitrile (1% v/v

sulphuric acid, 3% v/v triethylamine). A "V" section platinum anode was placed across the slide with the sharp edge resting against the derivatised surface. The cathode was a platinum wire held 10 mm above the anode and parallel to it. Voltage varying from 0.15 to 5 V/mm was applied in pulses between the electrodes for periods varying from 1 to 60 seconds. After each pulse the electrodes were moved along the slide to a new location. Removal of the dimethyoxytrityl groups by the acid generated at the anode exposed 5' hydroxyls on the thymidines which were reacted with (1-^{14}C) acetic anhydride. The slide was exposed to a PhosphorImager screen. The scan showed fine lines of radioactivity running across the slide where the anode had made contact. The intensity of the lines increased with applied voltage, reaching a maximum at 4 V/mm for 1 sec. The peak intensity corresponded to that measured on a control slide which had been treated with dichloroacetic acid under standard conditions for removing the dimethoxytrityl group. The width of the stripes was less than 0.5 mm.

In addition to achieving the desired result of narrow stripes, this example illustrates one advantage of the fine control that electrochemical generation of reagents makes possible, which in this case could lead to much faster reaction cycles. Acid deprotection of dimethoxytrityl groups takes 100 seconds using conventional chemistry; the reaction is carried out slowly because of the difficulty of timing the necessary change of solutions accurately. By contrast, it is easy to apply precisely regulated current in very short pulses and the corresponding electrolytic deprotection was complete in one second.

To make a complete array of oligonucleotides of every possible sequence using the simple protocol described in the caption to Fig. 1, it would be an

advantage to be able to have an array of linear electrodes permanently in place; the order of coupling different bases would be determined by switching the appropriate line in sequence.

The following example demonstrates such a device and illustrates some of its important features.

Example 2

The surface of a glass microscope slide was derivatised with an aliphatic chain bearing a primary and secondary hydroxyl group (Southern and Maskos, 1988). These groups were reacted with (1-^{14}C) acetic anhydride to form acetate esters. An electrode array comprising four parallel platinum strips (0.25 mm wide at 1 mm centres, embedded in epoxy resin and machined to a flat surface) was placed across the slide, with the two surfaces in contact. A small volume of triethylammonium sulphate in acetonitrile (1% v/v sulphuric acid, 3% v/v triethylamine) was run between the array and the slide so that the solution formed a film 5-10 µm in thickness. Electrodes 1, 2 and 4 of the array were connected to a DC supply as cathodes and number 3 was connected as an anode. Pulses of 5 and 10 V/mm were applied between the electrodes for periods varying from 1 to 10 seconds. After each pulse, the electrodes were moved along the slide to a new location. Removal of the (1-^{14}C) acetate groups by the acid generated at the anode was seen by exposing the slide to a PhosphorImager screen. The scan showed fine lines clear of radioactivity running across the slide where the anode had made contact. The width of the lines at half height was ca. 200 µm. Some apparent broadening of the band is caused by the detection system; the PhosphorImager screen has a grain size of 100 µm. Short pulses at low voltages gave a lower extent of deprotection than longer pulses at higher

voltage. The acetate was completely removed by a pulse of 10 V/mm for 5-10 sec.

Taken together, the two examples illustrate the versatility of the method: dimethoxytrityl groups are highly sensitive to acid whereas acetate esters are only moderately labile. Optimum conditions for removing these groups, using the same electrolyte, were achieved by varying the current intensity and the time of the electrolytic pulse. This shows how different regions of a surface can be modified in a different way simply by applying different potentials to the relevant electrodes, and how sequential steps in layering a surface, or in a complex synthesis, can be carried out without repositioning the array.

Similar procedures can be used to make oligomers other than oligonucleotides, for example oligopeptides or oligosaccharides; the standard synthetic chemistry for synthesising oligopeptides, which uses acid labile protecting groups is appropriate for this method. The principles are not confined to making oligomers of similar subunits, but could be applied to any chemical synthesis, and also to the modification of materials.

The second example illustrates the high resolution and definition which results from the barriers to diffusion created by the electric field. The sulphate ions which accumulate close to the anode are held away from the flanking cathodes by charge repulsion. The action of the acid generated by the sulphate ions is confined to the region of the slide which is directly opposite the anode.

The advantages of the electrolytic procedure over the alternatives of photodeprotection, masked coupling and micromanipulation are readily apparent.

Example 3

The fabrication of a 16 element microelectrode array has been achieved and this prototype has been used to demonstrate the viability of electrochemical patterning using electrodes ≈ 250 μm wide. In the second stage of development this design has been reduced in size and the number of microelectrodes increased to 256. This new array is currently in production and tests to evaluate its performance will commence in the near future.

Method

The primary requirements of the "first generation" microelectrode array are firstly, that it contains a minimum of 256 individual electrodes, each 50 - 100 μm wide and ≈ 50 mm in length and secondly that the array is uniformly flat and resistant to both mechanical wear and chemical attack. Two different approaches to fabrication have been investigated: in one the pattern is created by metallic deposition through a mask; in the other the pattern is cut out by detailed mechanical abrasion following a uniform metallic deposition.

i) **Masking**

Four different arrays each comprised of 16 microelectrodes were designed, drawn and etched into copper. The major problems with the fabrication of microelectrode arrays is that of "fan out", the electrical connection of many closely localised electrodes to an external device, but due to the small number of electrodes in these initial designs it was possible to incorporate a suitable "fan" arrangement on the mask so that the final array would be compatible with a standard circuit board connector. These masks have been successfully used to produce 16 element arrays with 200 μm electrode widths.

ii) Mechanical cutting

This is a simple but effective method of isolating electrodes by using a narrow blade diamond saw to cut shallow groves in a substrate coated with a thin metallic film. Electrode widths of ≈ 250 µm and separation of ≈ 250 µm have been routinely produced by this method. The problem of "fan out" however cannot be overcome during definition of the electrode pattern. This problem has been solved by connecting each electrode in turn to a printed circuit board with 50 µm gold wire using an impact bonder and embedding the fragile connections in epoxy resin. Manufacture of even smaller arrays by this method is presently underway using specialised diamond saws capable of defining 50 µm wide grooves.

Both these approaches are capable of producing microelectrode arrays suitable for initial testing. Further reduction in size will rule out mechanical cutting though we favour this as a method of fabrication because it is quick and inexpensive. Furthermore, the shallow channels cut between the electrodes using this method may provide a convenient irrigation system for the electrolyte and other reagents used in the production of the chemical sensors.

Materials

The main materials problem was that of securely bonding an inert metal to an unreactive insulating substrate. This problem has been overcome by depositing ≈ 1µm of platinum by electron-beam evaporation onto polished alumina (99.99% Bioceramic grade) followed by an anneal at 1100°C for 4 hours. At elevated temperatures the platinum diffuses down the grain boundaries of the alumina to form a very adherent coating which has proved to be resistant to both

mechanical wear and chemical attack.

Tests of the electrode array

The electrode array was first tested using a pH indicator system and shown to produce sharp lines of acid opposite electrodes connected as anodes, and then used in the following experiment. A 5′-O-trityl thymidine-3′-phosphate residue was coupled through the phosphate to a microscope slide which was then clamped against the Pt coated side of the electrode array. Electrolyte (0.01% H_2SO_4, 0.03% Et_3N in acetonitrile) was introduced between the slide and the array. 3 V DC was applied for 20, 40 and 80 sec. between single electrodes connected as anodes and the rest of the array which were connected as cathodes. The current, which was initially 30 µA, fell rapidly to 12 µA after about 2 sec. and then decreased slowly.

Hydroxyl groups exposed by acid hydrolysis of the trityl groups were detected by transferring ^{32}P from y-^{32}P-ATP to the hydroxyl groups catalysed by polynucleotide kinase. The isotope was detected in a Molecular Dynamics Phosphor Imager. The image shows clear lines where acid generated at the anode had deprotected the hydroxyl groups. The intensity increased with pulse time and at 80 sec. reached a level equivalent to that produced by standard deprotection in 3% TCA, which is known to be more than 99% complete.

As shown in Figure 2a), an array of electrodes is based on a block 10 of alumina, the upper surface of which carries a deposited layer 12 of platinum. Grooves 14 have been cut through this top coating into the alumina block, resulting in an array of parallel electrodes 16. The width of each electrode, and of each groove is approximately 250 µm. For the experiment of Example 3, a microscope slide 18

was used, the lower surface of which carried a uniform coating of 5′-O-trityl-thymidine-3′-phosphate residues coupled through the phosphate to the slide.

In Figure 2b), the slide has been clamped against the platinum coated side of the electrode array.

Figure 2c) shows the effect of connecting up alternate electrodes 16 as anode and cathode respectively. Et_3N^+ ions are generated at the cathodes 16a in the electrolyte 20. H^+ and SO_4^{--} ions are generated at the anode 16b in the electrolyte 20. As shown in Figure 2d), these H^+ ions cause deprotection of the thymidine-3′-phosphate residues adjacent the anode.

Figure 3 is a profile of the radioactive counts generated along three lines of deprotection, in the experiment of Example 3. The peak on the left was deprotected for 20 sec. and the other two for 40 and 80 sec. respectively. Detection of the ^{32}P by phosphor imaging degrades the image by shine from the radioisotope, accounting for much of the spread of the lines from the expected width of about 250 μm.

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CLAIMS

1. A method of electrochemically treating a region of a surface, which method comprises providing an electrolyte overlying the surface and an electrode adjacent the region of the surface to be treated and in contact with the electrolyte, and altering the potential of the electrode so as to deposit or remove or chemically modify a substance on the surface at the region adjacent the electrode.

2. A method as claimed in claim 1, wherein there is provided an array of electrodes adjacent the surface and in contact with the electrolyte, and the potential of one or more of the electrodes of the array is altered so as to deposit or chemically modify a substance on the surface at the regions adjacent those electrodes.

3. A method as claimed in claim 2, wherein one or more of the electrodes of the array are used as counter-electrodes.

4. A method as claimed in claim 3 wherein, for the purpose of performing several electrochemical treatments in sequence, the electrodes of the array are connected up so that each treatment is performed by altering the potential of a chosen set of one or more of the electrodes of the array.

5. A method as claimed in any one of claims 1 to 4, wherein the or each electrochemical treatment is performed by connecting the or each electrode as anode at a potential to remove an acid labile protecting group from a substance on the surface.

6. A method as claimed in claim 5, wherein the or each electrochemical treatment is performed in the

course of a stepwise chemical synthesis.

7. A method of synthesising a set of oligomers on a surface, by the steps of:-

a) providing a surface carrying a protected monomer, an electrolyte overlying the surface and one or more electrodes adjacent regions of the surface and in contact with the electrolyte,

b) using the method of any one of claims 1 to 6 to remove the protecting group from the monomer at one or more chosen regions of the surface,

c) depositing another protected monomer at those regions of the surface from which protecting group has been removed,

d) and repeating steps b) and c), while varying the region chosen in step b), so as to synthesise a set of oligomers on the surface.

8. A method as claimed in claim 7, wherein the oligomers are oligonucleotides.

9. A method as claimed in any one of claims 1 to 8, wherein the surface is electrically insulating.

10. An array of electrodes, suitable for use in the method of any one of claims 1 to 9, comprising a block of insulating material having a surface, and deposits of electrically conducting material spaced apart in an array on the surface, each deposit being provided with electrical connecting means for altering its potential.

11. An array as claimed in claim 10, wherein the block of insulating material is of an oxide ceramic and the deposits of electrically conducting material are of a noble metal.

12. An array as claimed in claim 10 or claim 11, wherein the deposits of electrically conducting material are in the form of parallel lines.

13. An array as claimed in claim 12, wherein the parallel lines are spaced apart by no more than 0.5 mm.

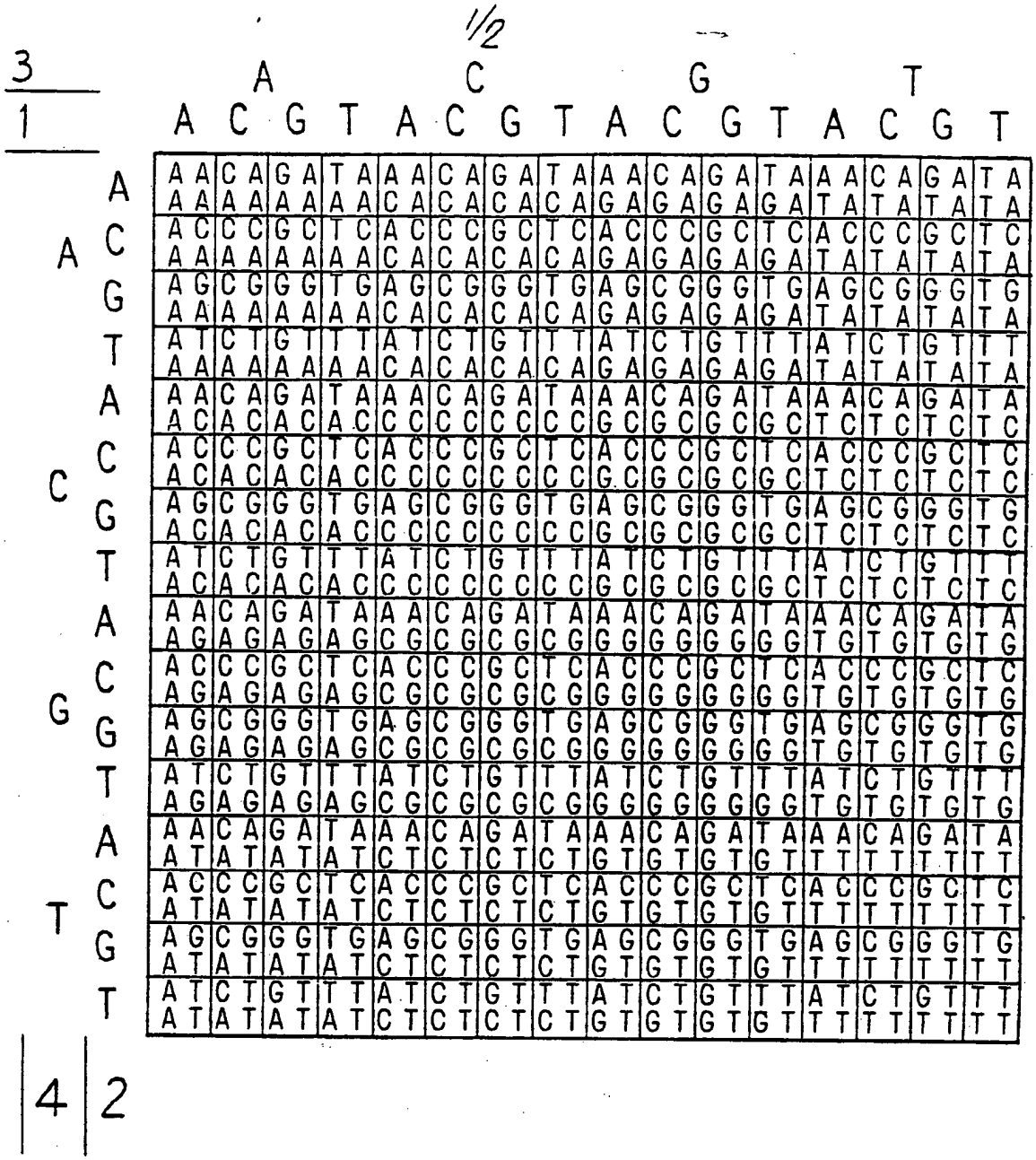

1/2
3
1
A C G T A C G T A C G T A C G T
A
C
G
T
4
2

FIG. 1

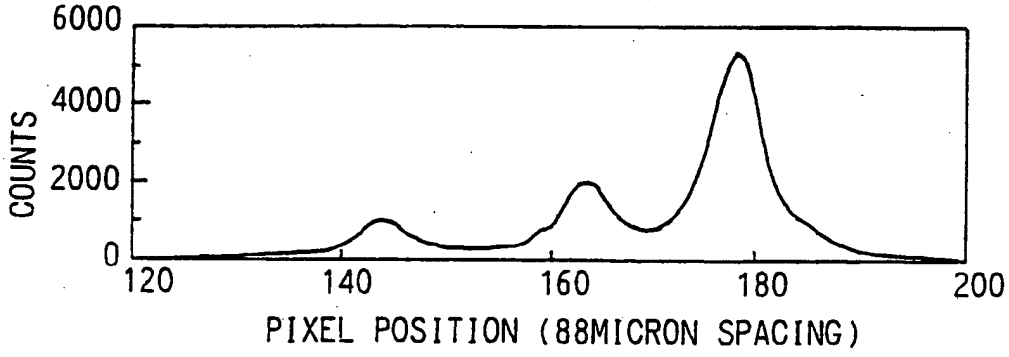

6000
4000
2000
0
COUNTS
120
140
160
180
200
PIXEL POSITION (88MICRON SPACING)

FIG. 3

FIG. 2(a)

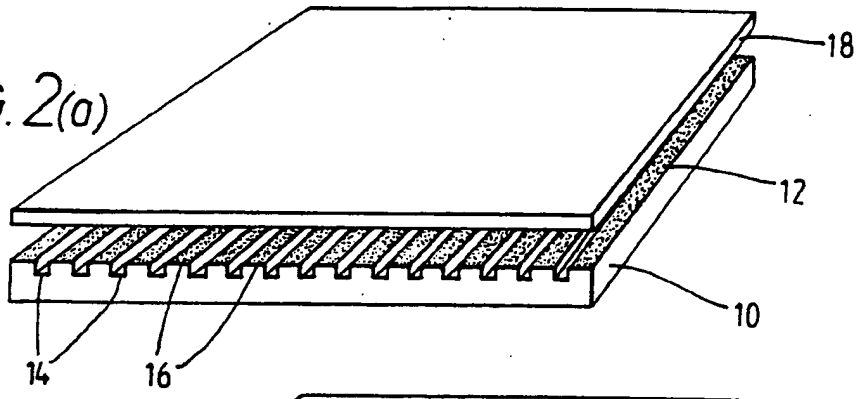


FIG. 2(b)

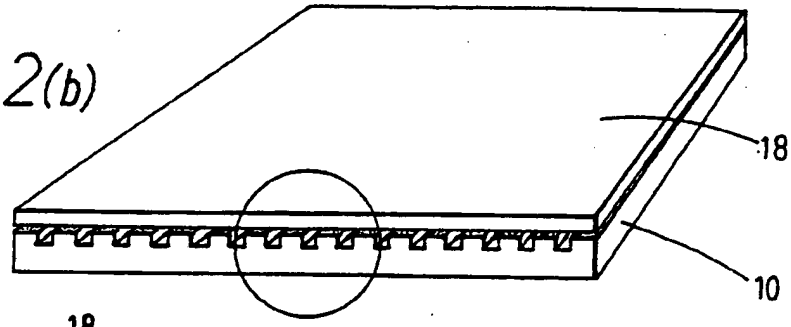


FIG. 2(c)

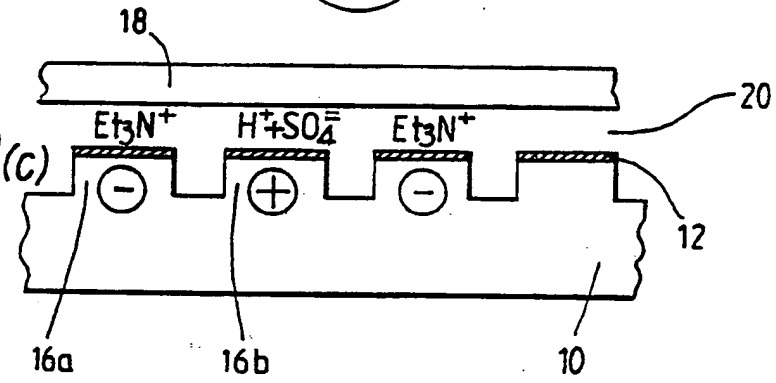
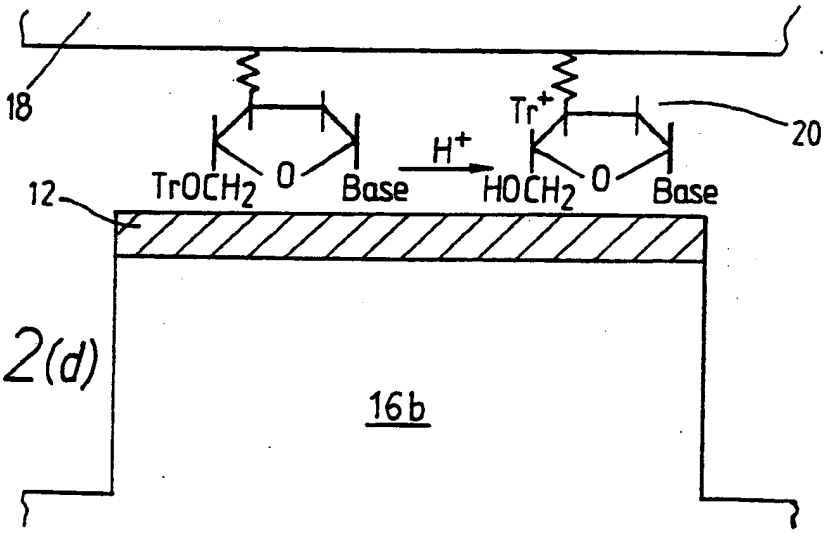


FIG. 2(d)



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00857

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 25 D 5/02,C 25 D 5/18,C 07 H 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 25 D,C 25 B,C 25 F,C 07 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 4 466 864 (BACON et al.) 21 August 1984 (21.08.84), abstract; fig. 3. --	1,4
A	PATENT ABSTRACTS OF JAPAN, unexamined applications, C field, vol. 9, no. 12, 18 January 1985 THE PATENT OFFICE JAPANESE GOVERNMENT, page 60 C 261, No. 59-162 290 (FUJITSU). ----	1,2,3, 4

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 July 1993	30 JUL 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUX e.h.

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/GB 93/00857 SAE 73148

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US A 4466864	21-08-84	keine - none - rien	

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(54) Title: SELF-ADDRESSABLE SELF-ASSEMBLING MICROELECTRONIC SYSTEMS AND DEVICES FOR MOLECULAR BIOLOGICAL ANALYSIS AND DIAGNOSTICS

(57) **Abstract**

A self-addressable, self-assembling microelectronic device is designed and fabricated to actively carry out and control multi-step and multiplex molecular biological reactions in microscopic formats. These reactions include nucleic acid hybridization, antibody/antigen reaction, diagnostics, and biopolymer synthesis. The device can be fabricated using both microlithographic and micro-machining techniques. The device can electronically control the transport and attachment of specific binding entities to specific micro-locations. The specific binding entities include molecular biological molecules such as nucleic acids and polypeptides. The device can subsequently control the transport and reaction of analytes or reactants at the addressed specific micro-locations. The device is able to concentrate analytes and reactants, remove non-specifically bound molecules, provide stringency control for DNA hybridization reactions, and improve the detection of analytes. The device can be electronically replicated.

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DESCRIPTION

SELF-ADDRESSABLE SELF-ASSEMBLING MICROELECTRONIC SYSTEMS AND DEVICES FOR MOLECULAR BIOLOGICAL ANALYSIS AND DIAGNOSTICS

Field of the Invention

This invention pertains to the design, fabrication, and uses of a self-addressable, self-assembling microelectronic system which can actively carry out and control multi-step and multiplex reactions in microscopic formats. In particular, these reactions include molecular biological reactions, such as nucleic acid hybridizations, antibody/antigen reactions, clinical diagnostics, and biopolymer synthesis.

Background of the Invention

Molecular biology comprises a wide variety of techniques for the analysis of nucleic acid and protein, many of which form the basis of clinical diagnostic assays. These techniques include nucleic acid hybridization analysis, restriction enzyme analysis, genetic sequence analysis, and separation and purification of nucleic acids and proteins (See, e.g., J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

Most molecular biology techniques involve carrying out numerous operations (e.g., pipetting) on a large number of samples. They are often complex and time consuming, and generally require a high degree of accuracy. Many a technique is limited in its application by a lack of sensitivity, specificity, or reproducibility. For example, problems with sensitivity and specificity have so far limited the application of nucleic acid hybridization.

Nucleic acid hybridization analysis generally involves the detection of a very small numbers of specific target nucleic acids (DNA or RNA) with probes among a large amount of non-target nucleic acids. In order to keep high specificity, hybridization is normally carried out under the most stringent condition, achieved through a combination of temperature, salts, detergents, solvents, chaotropic agents, and denaturants.

Multiple sample nucleic acid hybridization analysis has been conducted on a variety of filter and solid support formats (see G. A. Beltz et al., in Methods in Enzymology, Vol. 100, Part B, R. Wu, L. Grossmam, K. Moldave, Eds., Academic Press, New York, Chapter 19, pp. 266-308, 1985). One format, the so-called "dot blot" hybridization, involves the non-covalent attachment of target DNAs to a filter, which are subsequently hybridized with a radioisotope labeled probe(s). "Dot blot" hybridization gained wide-spread use, and many versions were developed (see M. L. M. Anderson and B. D. Young, in Nucleic Acid Hybridization - A Practical Approach, B. D. Hames and S. J. Higgins, Eds., IRL Press, Washington DC, Chapter 4, pp. 73-111, 1985). It has been developed for multiple analysis of genomic mutations (D. Nanibhushan and D. Rabin, in EPA 0228075, July 8, 1987) and for the detection of overlapping clones and the construction of genomic maps (G. A. Evans, in US Patent #5,219,726, June 15, 1993).

Another format, the so-called "sandwich" hybridization, involves attaching oligonucleotide probes covalently to a solid support and using them to capture and detect multiple nucleic acid targets. (M. Ranki et al., Gene, 21, pp. 77-85, 1983; A. M. Palva, T. M. Ranki, and H. E. Soderlund, in UK Patent Application GB 2156074A, October 2, 1985; T. M. Ranki and H. E. Soderlund in US Patent # 4,563,419, January 7, 1986; A. D. B. Malcolm and J. A. Langdale, in PCT WO 86/03782, July 3, 1986;

Y. Stabinsky, in US Patent # 4,751,177, January 14, 1988; T. H. Adams et al., in PCT WO 90/01564, February 22, 1990; R. B. Wallace et al. 6 Nucleic Acid Res. 11, p. 3543, 1979; and B. J. Connor et al., 80 Proc. Natl. Acad. Sci. USA pp. 278-282, 1983).

Using the current nucleic acid hybridization formats and stringency control methods, it remains difficult to detect low copy number (i.e., 1-100,000) nucleic acid targets even with the most sensitive reporter groups (enzyme, fluorophores, radioisotopes, etc.) and associated detection systems (fluorometers, luminometers, photon counters, scintillation counters, etc.).

This difficulty is caused by several underlying problems associated with direct probe hybridization. The first and the most serious problem relates to the stringency control of hybridization reactions. Hybridization reactions are usually carried out under the most stringent conditions in order to achieve the highest degree of specificity. Methods of stringency control involve primarily the optimization of temperature, ionic strength, and denaturants in hybridization and subsequent washing procedures. Unfortunately, the application of these stringency conditions causes a significant decrease in the number of hybridized probe/target complexes for detection.

The second problem relates to the high complexity of DNA in most samples, particularly in human genomic DNA samples. When a sample is composed of an enormous number of sequences which are closely related to the specific target sequence, even the most unique probe sequence has a large number of partial hybridizations with non-target sequences.

The third problem relates to the unfavorable hybridization dynamics between a probe and its specific target. Even under the best conditions, most hybridization reactions are conducted with relatively low concentrations of probes and target molecules. In addition, a probe often

has to compete with the complementary strand for the target nucleic acid.

The fourth problem for most present hybridization formats is the high level of non-specific background signal. This is caused by the affinity of DNA probes to almost any material.

These problems, either individually or in combination, lead to a loss of sensitivity and/or specificity for nucleic acid hybridization in the above described formats. This is unfortunate because the detection of low copy number nucleic acid targets is necessary for most nucleic acid-based clinical diagnostic assays.

Because of the difficulty in detecting low copy number nucleic acid targets, the research community relies heavily on the polymerase chain reaction (PCR) for the amplification of target nucleic acid sequences (see M. A. Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, 1990). The enormous number of target nucleic acid sequences produced by the PCR reaction improves the subsequent direct nucleic acid probe techniques, albeit at the cost of a lengthy and cumbersome procedure.

A distinctive exception to the general difficulty in detecting low copy number target nucleic acid with a direct probe is the in-situ hybridization technique. This technique allows low copy number unique nucleic acid sequences to be detected in individual cells. In the in-situ format, target nucleic acid is naturally confined to the area of a cell (~20-50 μm^2) or a nucleus (~10 μm^2) at a relatively high local concentration. Furthermore, the probe/target hybridization signal is confined to a morphologically distinct area; this makes it easier to distinguish a positive signal from artificial or non-specific signals than hybridization on a solid support.

Mimicking the in-situ hybridization, new techniques are being developed for carrying out multiple sample nucleic acid hybridization analysis on micro-formatted multiplex or matrix devices (e.g., DNA chips) (see M. Barinaga, 253 Science, pp. 1489, 1991; W. Bains, 10 Bio/Technology, pp. 757-758, 1992). These methods usually attach specific DNA sequences to very small specific areas of a solid support, such as micro-wells of a DNA chip. These hybridization formats are micro-scale versions of the conventional "dot blot" and "sandwich" hybridization systems.

The micro-formatted hybridization can be used to carry out "sequencing by hybridization" (SBH) (see M. Barinaga, 253 Science, pp. 1489, 1991; W. Bains, 10 Bio/Technology, pp. 757-758, 1992). SBH makes use of all possible n-nucleotide oligomers (n-mers) to identify n-mers in an unknown DNA sample, which are subsequently aligned by algorithm analysis to produce the DNA sequence (R. Drmanac and R. Crkvenjakov, Yugoslav Patent Application #570/87, 1987; R. Drmanac et al., 4 Genomics, 114, 1989; Strezoska et al., 88 Proc. Natl. Acad. Sci. USA 10089, 1991; and R. Drmanac and R. B. Crkvenjakov, US Patent #5,202,231, April 13, 1993).

There are two formats for carrying out SBH. The first format involves creating an array of all possible n-mers on a support, which is then hybridized with the target sequence. The second format involves attaching the target sequence to a support, which is sequentially probed with all possible n-mers. Both formats have the fundamental problems of direct probe hybridizations and additional difficulties related to multiplex hybridizations.

Southern, United Kingdom Patent Application GB 8810400, 1988; E. M. Southern et al., 13 Genomics 1008, 1992, proposed using the first format to analyze or sequence DNA. Southern identified a known single point mutation using PCR amplified genomic DNA. Southern also

described a method for synthesizing an array of oligonucleotides on a solid support for SBH. However, Southern did not address how to achieve optimal stringency condition for each oligonucleotide on an array.

Fodor et al., 364 Nature, pp. 555-556, 1993, used an array of 1,024 8-mer oligonucleotides on a solid support to sequence DNA. In this case, the target DNA was a fluorescently labeled single-stranded 12-mer oligonucleotide containing only nucleotides A and C. 1 pmol (~6 x 10^{11} molecules) of the 12-mer target sequence was necessary for the hybridization with the 8-mer oligomers on the array. The results showed many mismatches. Like Southern, Fodor et al., did not address the underlying problems of direct probe hybridization, such as stringency control for multiplex hybridizations. These problems, together with the requirement of a large quantity of the simple 12-mer target, indicate severe limitations to this SBH format.

Concurrently, Drmanac et al., 260 Science 1649-1652, 1993, used the second format to sequence several short (116 bp) DNA sequences. Target DNAs were attached to membrane supports ("dot blot" format). Each filter was sequentially hybridized with 272 labeled 10-mer and 11-mer oligonucleotides. A wide range of stringency condition was used to achieve specific hybridization for each n-mer probe; washing times varied from 5 minutes to overnight, and temperatures from 0°C to 16°C. Most probes required 3 hours of washing at 16°C. The filters had to be exposed for 2 to 18 hours in order to detect hybridization signals. The overall false positive hybridization rate was 5% in spite of the simple target sequences, the reduced set of oligomer probes, and the use of the most stringent conditions available.

Fodor et al., 251 Science 767-773, 1991, used photolithographic techniques to synthesize oligonucleotides on a matrix. Pirrung et al., in US Patent

5,143,854, September 1, 1992, teach large scale photolithographic solid phase synthesis of polypeptides in an array fashion on silicon substrates.

In another approach of matrix hybridization, Beattie et al., in The 1992 San Diego Conference: Genetic Recognition, November, 1992, used a microrobotic system to deposit micro-droplets containing specific DNA sequences into individual microfabricated sample wells on a glass substrate. The hybridization in each sample well is detected by interrogating miniature electrode test fixtures, which surround each individual microwell with an alternating current (AC) electric field.

Regardless of the format, current micro-scale DNA hybridization and SBH approaches do not overcome the underlying physical problems associated with direct probe hybridization reactions. They require very high levels of relatively short single-stranded target sequences or PCR amplified DNA, and produce a high level of false positive hybridization signals even under the most stringent conditions. In the case of multiplex formats using arrays of short oligonucleotide sequences, it is not possible to optimize the stringency condition for each individual sequence with any conventional approach because the arrays or devices used for these formats can not change or adjust the temperature, ionic strength, or denaturants at an individual location, relative to other locations. Therefore, a common stringency condition must be used for all the sequences on the device. This results in a large number of non-specific and partial hybridizations and severely limits the application of the device. The problem becomes more compounded as the number of different sequences on the array increases, and as the length of the sequences decreases. This is particularly troublesome for SBH, which requires a large number of short oligonucleotide probes.

Nucleic acids of different size, charge, or conformation are routinely separated by electrophoresis techniques which can distinguish hybridization species by their differential mobility in an electric field. Pulse field electrophoresis uses an arrangement of multiple electrodes around a medium (e.g., a gel) to separate very large DNA fragments which cannot be resolved by conventional gel electrophoresis systems (see R. Anand and E. M. Southern in Gel Electrophoresis of Nucleic Acids - A Practical Approach, 2 ed., D. Rickwood and B. D. Hames Eds., IRL Press, New York, pp. 101-122, 1990).

Pace, US Patent #4,908,112, March 13, 1990, teaches using micro-fabrication techniques to produce a capillary gel electrophoresis system on a silicon substrate. Multiple electrodes are incorporated into the system to move molecules through the separation medium within the device.

Soane and Soane, US Patent 5,126,022, June 30, 1992, teach that a number of electrodes can be used to control the linear movement of charged molecules in a mixture through a gel separation medium contained in a tube. Electrodes have to be installed within the tube to control the movement and position of molecules in the separation medium.

Washizu, M. and Kurosawa, O., 26 IEEE Transactions on Industry Applications 6, pp. 1165-1172, 1990, used high-frequency alternating current (AC) fields to orient DNA molecules in electric field lines produced between microfabricated electrodes. However, the use of direct current (DC) fields is prohibitive for their work. Washizu 25 Journal of Electrostatics 109-123, 1990, describes the manipulation of cells and biological molecules using dielectrophoresis. Cells can be fused and biological molecules can be oriented along the electric fields lines produced by AC voltages between the microelectrode structures. However, the dielectrophoresis process requires a very high frequency AC (1 MHz) voltage

and a low conductivity medium. While these techniques can orient DNA molecules of different sizes along the AC field lines, they cannot distinguish between hybridization complexes of the same size.

As is apparent from the preceding discussion, numerous attempts have been made to provide effective techniques to conduct multi-step, multiplex molecular biological reactions. However, for the reasons stated above, these techniques have been proved deficient. Despite the long-recognized need for effective technique, no satisfactory solution has been proposed previously.

Summary of the Invention

The present invention relates to the design, fabrication, and uses of a self-addressable self-assembling microelectronic system and device which can actively carry out controlled multi-step and multiplex reactions in microscopic formats. These reactions include, but are not limited to, most molecular biological procedures, such as nucleic acid hybridization, antibody/antigen reaction, and related clinical diagnostics. In addition, the claimed device is able to carry out multi-step combinational biopolymer synthesis, including, but not limited to, the synthesis of different oligonucleotides or peptides at specific micro-locations.

The claimed device is fabricated using both micro-lithographic and micro-machining techniques. The device has a matrix of addressable microscopic locations on its surface; each individual micro-location is able to electronically control and direct the transport and attachment of specific binding entities (e.g., nucleic acids, antibodies) to itself. All micro-locations can be addressed with their specific binding entities. Using this device, the system can be self-assembled with minimal outside intervention.

The device is able to control and actively carry out a variety of assays and reactions. Analytes or reactants can be transported by free field electrophoresis to any specific micro-location where the analytes or reactants are effectively concentrated and reacted with the specific binding entity at said micro-location. The sensitivity for detecting a specific analyte or reactant is improved because of the concentrating effect. Any un-bound analytes or reactants can be removed by reversing the polarity of a micro-location. Thus, the device also improves the specificity of assays and reactions.

The device provides independent stringency control for hybridization reactions at specific micro-locations. Thus all the micro-locations on the matrix can have different stringency conditions at the same time, allowing multiple hybridizations to be conducted at optimal conditions.

The device also facilitates the detection of hybridized complexes at each micro-location by using an associated optical (fluorescent or spectrophotometric) imaging detector system or an integrated sensing component.

In addition, the active nature of the device allows complex multi-step reactions to be carried out with minimal outside physical manipulations. If desired, a master device addressed with specific binding entities can be electronically replicated or copied to another base device.

Thus, the claimed device can carry out multi-step and multiplex reactions with complete and precise electronic control, preferably with a micro-processor. The rate, specificity, and sensitivity of multi-step and multiplex reactions are greatly improved at specific micro-locations of the claimed device.

The present invention overcomes the limitations of the arrays and devices for multi-sample hybridizations described in the background of the invention. Previous

methods and devices are functionally passive regarding the actual hybridization process. While sophisticated photolithographic techniques were used to make an array, or microelectronic sensing elements were incorporated for detection, previous devices did not control or influence the actual hybridization process. They are not designed to actively overcome any of the underlying physical problems associated with hybridization reactions.

This invention may utilize micro-locations of any size or shape consistent with the objective of the invention. In the preferred embodiment of the invention, micro-locations in the sub-millimeter range are used.

By "specific binding entity" is generally meant a biological or synthetic molecule that has specific affinity to another molecule, through covalent bonding or non-covalent bonding. Preferably, a specific binding entity contains (either by nature or by modification) a functional chemical group (primary amine, sulfhydryl, aldehyde, etc.), a common sequence (nucleic acids), an epitope (antibodies), a hapten, or a ligand, that allows it to covalently react or non-covalently bind to a common functional group on the surface of a micro-location. Specific binding entities include, but are not limited to: deoxyribonucleic acids (DNA), ribonucleic acids (RNA), synthetic oligonucleotides, antibodies, proteins, peptides, lectins, modified polysaccharides, synthetic composite macromolecules, functionalized nanostructures, synthetic polymers, modified/blocked nucleotides/nucleosides, modified/blocked amino acids, fluorophores, chromophores, ligands, chelates and haptens.

By "stringency control" is meant the ability to discriminate specific and non-specific binding interactions.

Thus, in a first aspect, the present invention features a device with an array of electronically self-addressable microscopic locations. Each microscopic

location contains an underlying working direct current (DC) micro-electrode supported by a substrate. The surface of each micro-location has a permeation layer for the free transport of small counter-ions, and an attachment layer for the covalent coupling of specific binding entities.

By "array" or "matrix" is meant an arrangement of locations on the device. The locations can be arranged in two dimensional arrays, three dimensional arrays, or other matrix formats. The number of locations can range from several to at least hundreds of thousands.

In a second aspect, this invention features a method for transporting the binding entity to any specific micro-location on the device. When activated, a micro-location can affect the free field electrophoretic transport of any charged functionalized specific binding entity directly to itself. Upon contacting the specific micro-location, the functionalized specific binding entity immediately becomes covalently attached to the attachment layer surface of that specific micro-location. Other micro-locations can be simultaneously protected by maintaining them at the opposite potential to the charged molecules. The process can be rapidly repeated until all the micro-locations are addressed with their specific binding entities.

By "charged functionalized specific binding entity" is meant a specific binding entity that is chemically reactive (i.e., capable of covalent attachment to a location) and carrying a net change (either positive or negative).

In a third aspect, this inventions features a method for concentrating and reacting analytes or reactants at any specific micro-location on the device. After the attachment of the specific binding entities, the underlying microelectrode at each micro-location continues to function in a direct current (DC) mode. This unique feature allows relatively dilute charged analytes or

reactant molecules free in solution to be rapidly transported, concentrated, and reacted in a serial or parallel manner at any specific micro-locations which are maintained at the opposite charge to the analyte or reactant molecules. Specific micro-locations can be protected or shielded by maintaining them at the same charge as the analytes or reactants molecules. This ability to concentrate dilute analyte or reactant molecules at selected micro-locations greatly accelerates the reaction rates at these micro-locations.

When the desired reaction is complete, the microelectrode potential can be reversed to remove non-specific analytes or unreacted molecules from the micro-locations.

Specific analytes or reaction products may be released from any micro-location and transported to other locations for further analysis; or stored at other addressable locations; or removed completely from the system.

The subsequent analysis of the analytes at the specific micro-locations is also greatly improved by the ability to repulse non-specific entities from these locations.

In a fourth aspect, this invention features a method for improving stringency control of nucleic acid hybridization reactions, comprising the steps of:

-rapidly concentrating dilute target DNA and/or probe DNA sequences at specific micro-location(s) where hybridization is to occur;

-rapidly removing non-specifically bound target DNA sequences from specific micro-location(s) where hybridization has occurred;

-rapidly removing competing complementary target DNA sequences from specific micro-location(s) where hybridization has occurred;

-raising electric potential to remove partially hybridized DNA sequences (more than one base mis-match);

-adjusting electric potential to improve the resolution of single mis-match hybridizations (e.g., to identify point mutations);

-applying independent electric potential control to individual hybridization events occurring in the same bulk solution; and

-using electric potential control to improve hybridization of un-amplified target DNA sequences to arrays of capture oligonucleotide probes.

In a fifth aspect, this invention features a method for synthesizing biopolymers at micro-locations.

In a sixth aspect, this invention features a method for replicating a master device.

In a seventh aspect, this invention features methods for detecting and analyzing reactions that have occurred at the addressed micro-locations using self-addressed microelectronic devices with associated optical, optoelectronic or electronic detection systems or self-addressed microelectronic devices with integrated optical, optoelectronic or electronic detection systems.

Brief Description of the Drawings

FIGURE 1 is the cross-section of three self-addressable micro-locations fabricated using microlithographic techniques.

FIGURE 2 is the cross-section of a microlithographically fabricated micro-location.

FIGURE 3 is a schematic representation of a self-addressable 64 micro-location chip which was actually fabricated, addressed with oligonucleotides, and tested.

FIGURE 4 shows particular attachment chemistry procedure which allows rapid covalent coupling of specific oligonucleotides to the attachment surface of a micro-location.

FIGURE 5 is a blown-up schematic diagram of a micro-machined 96 micro-locations device.

FIGURE 6 is the cross-section of a micro-machined device.

FIGURE 7 shows the mechanism the device uses to electronically concentrate analyte or reactant molecules at a specific micro-location.

FIGURE 8 shows the self-directed assembly of a device with three specific oligonucleotide binding entities (SSO-A, SSO-B, and SSO-C).

FIGURE 9 shows an electronically controlled hybridization process with sample/target DNA being concentrated at micro-locations containing specific DNA capture sequences.

FIGURE 10 shows an electronically directed serial hybridization process.

FIGURE 11 shows the electronic stringency control (ESC) of a hybridization process for determining single point mutations.

FIGURE 12 shows a scheme for the detection of hybridized DNA without using labeled DNA probe, i.e., electronically controlled fluorescent dye detection process.

FIGURE 13 shows a scheme of electronically controlled replication of devices.

FIGURE 14 shows a scheme of electronically directed combinatorial synthesis of oligonucleotides.

Detailed Description of the Invention

The devices and the related methodologies of this invention allow important molecular biology and diagnostic reactions to be carried out under complete electronic control. The basic concept of this invention is a micro-electronic device with specially designed addressable microscopic locations. Each micro-location has a derivatized surface for the covalent attachment of specific binding entities (i.e., an attachment layer), a permeation layer, and an underlying direct current (DC) micro-

electrode. After the initial fabrication of the basic microelectronic structure, the device is able to self-direct the addressing of each specific micro-location with specific binding entities. The self-addressed device is subsequently able to actively carry out multi-step, combinatorial, and multiplex reactions at any of its micro-locations. The device is able to electronically direct and control the rapid movement and concentration of analytes and reactants to or from any of its micro-locations. The ability of the device to electronically control the dynamic aspects of various reactions provides a number of new and important advantages and improvements.

The concepts and embodiments of this invention are described in three sections. The first section, "Design and Fabrication of the Basic Devices," describes the design of the basic underlying microelectronic device and the fabrication of the device using microlithographic and micromachining techniques. The second section, "Self-Directed Addressing of the Devices," describes the self-addressing and self-assembly of the device, specifically the rapid transport and attachment of specific binding entities to each micro-location. The third section, "Applications of the Devices," describes how the device provides electronic control of various multi-step, combinatorial, and multiplex reactions. This section also describes the various uses and applications of the device.

(1) DESIGN AND FABRICATION OF THE BASIC DEVICES

In order for a device to carry out multi-step and multiplex reactions, its crucial electronic components must be able to maintain active operation in aqueous solutions. To satisfy this requirement, each micro-location must have an underlying functioning DC mode micro-electrode. Other considerations for the design and fabrication of a device include, but are not limited to, materials compatibilities, nature of the specific binding

entities and the subsequent reactants and analytes, and the number of micro-locations.

By "a functioning DC mode micro-electrode" is meant a micro-electrode biased either positively or negatively, operating in a direct current mode (either continuous or pulse), which can affect or cause the free field electrophoretic transport of charged specific binding entities, reactants, or analytes to or from any location on the device, or in the sample solution.

Within the scope of this invention, the free field electrophoretic transport of molecules is not dependent on the electric field produced being bounded or confined by dielectrical material.

A device can be designed to have as few as two addressable micro-locations or as many as hundreds of thousands of micro-locations. In general, a complex device with a large number of micro-locations is fabricated using microlithography techniques. Fabrication is carried out on silicon or other suitable substrate materials, such as glass, silicon dioxide, plastic, or ceramic materials. These microelectronic "chip" designs would be considered large scale array or multiplex analysis devices. A device with a small number of micro-locations would be fabricated using micro-machining techniques.

Addressable micro-locations can be of any shape, preferably round, square, or rectangular. The size of an addressable micro-location can be of any size, preferably range from sub-micron (~0.5 μm) to several centimeters (cm), with 5 μm to 100 μm being the most preferred size range for devices fabricated using microlithographic techniques, and 100 μm to 5 millimeters being the most preferred size range for devices fabricated using the micro-machining techniques. To make micro-locations smaller than the resolution of microlithographic methods would require techniques such as electron beam

lithography, ion beam lithography, or molecular beam epitaxy. While microscopic locations are desirable for analytical and diagnostic type applications, larger addressable locations (e.g., larger than 2 mm) are desirable for preparative scale biopolymer synthesis.

After micro-locations have been created by using microlithographic and/or micro-machining techniques, chemical techniques are used to create the specialized attachment and permeation layers which would allow the DC mode micro-electrodes under the micro-locations to: (1) affect or cause the free field electrophoretic transport of specific (charged) binding entities from any location; (2) concentrate and covalently attach the specific binding entities to the specially modified surface of the specific micro-location; and (3) continue to actively function in the DC mode after the attachment of specific binding entities so that other reactants and analytes can be transported to or from the micro-locations.

DESIGN PARAMETERS (MICROLITHOGRAPHY)

Figure 1 shows a basic design of self-addressable micro-locations fabricated using microlithographic techniques. The three micro-locations (10) (ML-1, ML-2, ML-3) are formed on the surface of metal sites (12) which have been deposited on an insulator layer/base material. The metal sites (12) serve as the underlying micro-electrode structures (10). An insulator material separates the metal sites (12) from each other. Insulator materials include, but are not limited to, silicon dioxide, glass, resist, rubber, plastic, or ceramic materials.

Figure 2 shows the basic features of an individual micro-location (10) formed on a microlithographically produced metal site (12). The addressable micro-location is formed on the metal site (12), and incorporates an oxidation layer (20), a permeation layer (22), an

attachment layer (24), and a binding entity layer (26). The metal oxide layer provides a base for the covalent coupling of the permeation layer. The permeation layer provides spacing between the metal surface and the attachment/binding entity layers and allows solvent molecules, small counter-ions, and gases to freely pass to and from the metal surface. The thickness of the permeation layer for microlithographically produced devices can range from approximately 1 nanometers (nm) to 10 microns (μm), with 2 nm to 1 μm being the most preferred. The attachment layer provides a base for the covalent binding of the binding entities. The thickness of the attachment layer for microlithographically produced devices can range from 0.5 nm to 1 μm, with 1 nm to 200 nm being the most preferred. In some cases, the permeation and attachment layers can be formed from the same material. The specific binding entities are covalently coupled to the attachment layer, and form the specific binding entity layer. The specific binding entity layer is usually a mono-layer of the specific binding molecules. However, in some cases the binding entity layer can have several or even many layers of binding molecules.

Certain design and functional aspects of the permeation and attachment layer are dictated by the physical (e.g., size and shape) and the chemical properties of the specific binding entity molecules. They are also dictated to some extent by the physical and chemical properties of the reactant and analyte molecules, which will be subsequently transported and bound to the micro-location. For example, oligonucleotide binding entities can be attached to one type of micro-location surface without causing a loss of the DC mode function, i.e., the underlying micro-electrode can still cause the rapid free field electrophoretic transport of other analyte molecules to or from the surface to which the oligonucleotide binding entities are attached. However, if large globular

protein binding entities (e.g., antibodies) are attached to the same type of surface, they may effectively insulate the surface and cause a decrease or a complete loss of the DC mode function. Appropriate modification of the attachment layer would have to be carried out so as to either reduce the number of large binding entities (e.g., large globular proteins) or provide spacing between the binding entities on the surface.

The spacing between micro-locations is determined by the ease of fabrication, the requirement for detector resolution between micro-locations, and the number of micro-locations desired on a device. However, particular spacings between micro-locations, or special arrangement or geometry of the micro-locations is not necessary for device function, in that any combination of micro-locations (i.e., underlying micro-electrodes) can operate over the complete device area. Nor is it necessary to enclose the device or confine the micro-locations with dielectric boundaries. This is because complex electronic field patterns or dielectric boundaries are not required to selectively move, separate, hold, or orient specific molecules in the space or medium between any of the electrodes. The device accomplishes this by attaching the specific binding molecules and subsequent analytes and reactants to the surface of an addressable micro-location. Free field electrophoretic propulsion provides for the rapid and direct transport of any charged molecule between any and all locations on the device.

As the number of micro-locations increases beyond several hundred, the complexity of the underlying circuitry of the micro-locations increases. In this case the micro-location grouping patterns have to be changed and spacing distances increased proportionally, or multi-layer circuitry can be fabricated into the basic device.

In addition to micro-locations which have been addressed with specific binding entities, a device will

contain some un-addressed, or plain micro-locations which serve other functions. These micro-locations can be used to store reagents, to temporarily hold reactants or analytes, and as disposal units for excess reactants, analytes, or other interfering components in samples. Other un-addressed micro-locations can be used in combination with the addressed micro-locations to affect or influence the reactions that are occurring at these specific micro-locations. These micro-locations add to intra-device activity and control. It is also possible for the micro-locations to interact and transport molecules between two separate devices. This provides a mechanism for loading a working device with binding entities or reactants from a storage device, and for copying or replicating a device.

Figure 3 shows a matrix type device containing 64 addressable micro-locations (30). A 64 micro-location device is a convenient design, which fits with standard microelectronic chip packaging components. Such a device is fabricated on a silicon chip substrate approximately 1.5 cm x 1.5 cm, with a central area approximately 750 μm x 750 μm containing the 64 micro-locations. Each micro-location (32) is approximately 50 μm square with 50 μm spacing between neighboring micro-locations. Connective circuitry for each individual underlying micro-electrode runs to an outside perimeter (10 mm x 10 mm) of metal contact pads (300 μm square) (34). A raised inner perimeter can be formed between the area with the micro-locations and the contact pads, producing a cavity which can hold approximately 2 to 10 microliters (μl) of a sample solution. The "chip" can be mounted in a standard quad package, and the chip contact pads (34) wired to the quad package pins. The packaged chip can then be plugged into a microprocessor controlled DC power supply and multimeter apparatus which can control and operate the device.

FABRICATION PROCEDURES (MICROLITHOGRAPHY)

Microlithography Fabrication Steps

General microlithographic or photolithographic techniques can be used for the fabrication of the complex "chip" type device which has a large number of small micro-locations. While the fabrication of devices does not require complex photolithography, the selection of materials and the requirement that an electronic device function actively in aqueous solutions does require special considerations.

The 64 micro-location device (30) shown in Figure 3 can be fabricated using relatively simple mask design and standard microlithographic techniques. Generally, the base substrate material would be a 1 to 2 centimeter square silicon wafer or a chip approximately 0.5 millimeter in thickness. The silicon chip is first overcoated with a 1 to 2 μm thick silicon dioxide (SiO_2) insulation coat, which is applied by plasma enhanced chemical vapor deposition (PECVD).

In the next step, a 0.2 to 0.5 μm metal layer (e.g., aluminum) is deposited by vacuum evaporation. In addition to aluminum, suitable metals for circuitry include gold, silver, tin, copper, platinum, palladium, carbon, and various metal combinations. Special techniques for ensuring proper adhesion to the insulating substrate materials (SiO_2) are used with different metals.

The chip is next overcoated with a positive photoresist (Shipley, Microposit AZ 1350 J), masked (light field) with the circuitry pattern, exposed and developed. The photosolubilized resist is removed, and the exposed aluminum is etched away. The resist island is now removed, leaving the aluminum circuitry pattern on the chip. This includes an outside perimeter of metal contact pads, the connective circuitry (wires), and the center array of micro-electrodes which serve as the underlying base for the addressable micro-locations.

Using PECVD, the chip is overcoated first with a 0.2 to 0.4 micron layer of SiO_2, and then with a 0.1 to 0.2 micron layer of silicon nitride (Si_3N_4). The chip is then covered with positive photoresist, masked for the contact pads and micro-electrode locations, exposed, and developed. Photosolubilized resist is removed, and the SiO_2 and Si_3N_4 layers are etched away to expose the aluminum contact pads and micro-electrodes. The surrounding island resist is then removed, the connective wiring between the contact pads and the micro-electrodes remains insulated by the SiO_2 and Si_3N_4 layers.

The SiO_2 and Si_3N_4 layers provide important properties for the functioning of the device. First, the second SiO_2 layer has better contact and improved sealing with the aluminum circuitry. It is also possible to use resist materials to insulate and seal. This prevents undermining of the circuitry due to electrolysis effects when the micro-electrodes are operating. The final surface layer coating of Si_3N_4 is used because it has much less reactivity with the subsequent reagents used to modify the micro-electrode surfaces for the attachment of specific binding entities.

Permeation and Attachment Layer Formation Steps

At this point the micro-electrode locations on the device are ready to be modified with a specialized permeation and attachment layer. This represents the most important aspect of the invention, and is crucial for the active functioning of the device. The objective is to create on the micro-electrode an intermediate permeation layer with selective diffusion properties and an attachment surface layer with optimal binding properties. The attachment layer should have from 10^5 to 10^7 functionalized locations per square micron (μm^2) for the optimal attachment of specific binding entities. However, the attachment of specific binding entities must not overcoat

or insulate the surface so as to prevent the underlying micro-electrode from functioning. A functional device requires some fraction (~ 5% to 25%) of the actual metal micro-electrode surface to remain accessible to solvent (H_2O) molecules, and to allow the diffusion of counter-ions (e.g., Na^+ and Cl^-) and electrolysis gases (e.g., O_2 and H_2) to occur.

The intermediate permeation layer must also allow diffusion to occur. Additionally, the permeation layer should have a pore limit property which inhibits or impedes the larger binding entities, reactants, and analytes from physical contact with the micro-electrode surface. The permeation layer keeps the active micro-electrode surface physically distinct from the binding entity layer of the micro-location.

In terms of the primary device function, this design allows the electrolysis reactions required for electrophoretic transport to occur on micro-electrode surface, but avoids adverse electrochemical effects to the binding entities, reactants, and analytes.

One preferred procedure for the derivatization of the metal micro-electrode surface uses aminopropyltriethoxy silane (APS). APS reacts readily with the oxide and/or hydroxyl groups on metal and silicon surfaces. APS provides a combined permeation layer and attachment layer, with primary amine groups for the subsequent covalent coupling of binding entities. In terms of surface binding sites, APS produces a relatively high level of functionalization (i.e., a large number of primary amine groups) on slightly oxidized aluminum surfaces, an intermediate level of functionalization on SiO_2 surfaces, and very limited functionalization of Si_3N_4 surfaces.

The APS reaction is carried out by treating the whole device (e.g., a chip) surface for 30 minutes with a 10% solution of APS in toluene at 50°C. The chip is then washed in toluene, ethanol, and then dried for one hour at

50°C. The micro-electrode metal surface is functionalized with a large number of primary amine groups (10^5 to 10^6 per square micron). Binding entities can now be covalently bound to the derivatized micro-electrode surface.

The APS procedure works well for the attachment of oligonucleotide binding entities. Figure 4 shows the mechanism for the attachment of 3'-terminal aldehyde derivatized oligonucleotides (40) to an APS functionalized surface (42). While this represents one of the preferred approaches, a variety of other attachment reactions are possible for both the covalent and non-covalent attachment of many types of binding entities.

DESIGN AND FABRICATION (MICRO-MACHINING)

This section describes how to use micro-machining techniques (e.g., drilling, milling, etc.) or non-lithographic techniques to fabricate devices. In general, these devices have relatively larger micro-locations (> 100 microns) than those produced by microlithography. These devices could be used for analytical applications, as well as for preparative type applications, such as biopolymer synthesis. Large addressable locations could be fabricated in three dimensional formats (e.g., tubes or cylinders) in order to carry a large amount of binding entities. Such devices could be fabricated using a variety of materials, including, but not limited to, plastic, rubber, silicon, glass (e.g., microchannelled, microcapillary, etc.), or ceramics. In the case of micro-machined devices, connective circuitry and larger electrode structures can be printed onto materials using standard circuit board printing techniques known to those skilled in the art.

Addressable micro-location devices can be fabricated relatively easily using micro-machining techniques. Figure 5 is a schematic of a representative 96 micro-location device. This micro-location device is fabricated

from a suitable material stock (2 cm x 4 cm x 1 cm), by drilling 96 proportionately spaced holes (1 mm in diameter) through the material. An electrode circuit board (52) is formed on a thin sheet of plastic material stock, which fit precisely over the top of the micro-location component (54). The underside of the circuit board contains the individual wires (printed circuit) to each micro-location (55). Short platinum electrode structures (~ 3-4 mm) (62) are designed to extended down into the individual micro-location chambers (57). The printed circuit wiring is coated with a suitable water-proof insulating material. The printed circuit wiring converges to a socket, which allows connection to a multiplex switch controller (56) and DC power supply (58). The device is partially immersed and operates in a common buffer reservoir (59).

While the primary function of the micro-locations in devices fabricated by micro-machining and microlithography techniques is the same, their designs are different. In devices fabricated by microlithography, the permeation and attachment layers are formed directly on the underlying metal micro-electrode. In devices fabricated by micro-machining techniques, the permeation and attachment layers are physically separated from their individual metal electrode structure (62) by a buffer solution in the individual chamber or reservoir (57) (see Figure 6). In micro-machined devices the permeation and attachment layers can be formed using functionalized hydrophilic gels, membranes, or other suitable porous materials.

In general, the thickness of the combined permeation and attachment layers ranges from 10 μm to 10 mm. For example, a modified hydrophilic gel of 26% to 35 % polyacrylamide (with 0.1% polylysine), can be used to partially fill (~ 0.5 mm) each of the individual micro-location chambers in the device. This concentration of gel forms an ideal permeation layer with a pore limit of from 2 nm

to 3 nm. The polylysine incorporated into the gel provides primary amine functional groups for the subsequent attachment of specific binding entities. This type of gel permeation layer allows the electrodes to function actively in the DC mode. When the electrode is activated, the gel permeation layer allows small counter-ions to pass through it, but the larger specific binding entity molecules are concentrated on the outer surface. Here they become covalently bonded to the outer layer of primary amines, which effectively becomes the attachment layer.

An alternative technique for the formation of the permeation and attachment layers is to incorporate into the base of each micro-location chamber a porous membrane material. The outer surface of the membrane is then derivatized with chemical functional groups to form the attachment layer. Appropriate techniques and materials for carrying out this approach are known to those skilled in the art.

The above description for the design and fabrication of a device should not be considered as a limit to other variations or forms of the basic device. Many variations of the device with larger or smaller numbers of addressable micro-locations are envisioned for different analytical and preparative applications. Variations of the device with larger addressable locations are envisioned for preparative biopolymer synthesis applications. Variations are also contemplated as electronically addressable and controllable reagent dispensers for use with other devices, including those produced by microlithographic techniques.

(2) SELF-DIRECTED ADDRESSING OF THE DEVICES

The claimed devices are able to electronically self-address each micro-location with a specific binding entity. The device itself directly affects or causes the

transport and attachment of specific binding entities to specific micro-locations. The device self-assembles itself in the sense that no outside process, mechanism, or equipment is needed to physically direct, position, or place a specific binding entity at a specific micro-location. This self-addressing process is both rapid and specific, and can be carried out in either a serial or parallel manner.

A device can be serially addressed with specific binding entities by maintaining the selected micro-location in a DC mode and at the opposite charge (potential) to that of a specific binding entity. All other micro-locations are maintained at the same charge as the specific binding entity. In cases where the binding entity is not in excess of the attachment sites on the micro-location, it is necessary to activate only one other micro-electrode to affect the electrophoretic transport to the specific micro-location. The specific binding entity is rapidly transported (in a few seconds, or preferably less than a second) through the solution, and concentrated directly at the specific micro-location where it immediately becomes covalently bonded to the special surface. The ability to electronically concentrate reactants or analytes (70) on a specific micro-location (72) is shown in Figure 7. All other micro-locations remain unaffected by that specific binding entity. Any unreacted binding entity is removed by reversing the polarity of that specific micro-location, and electrophoresing it to a disposal location. The cycle is repeated until all desired micro-locations are addressed with their specific binding entities. Figure 8 shows the serial process for addressing specific micro-locations (81, 83, 85) with specific oligonucleotide binding entities (82, 84, 86).

The parallel process for addressing micro-locations simply involves simultaneously activating a large number

(particular group or line) of micro-electrodes so that the same specific binding entity is transported, concentrated, and reacted with more than one specific micro-locations.

(3) APPLICATIONS OF THE DEVICES

Once a device has been self-addressed with specific binding entities, a variety of molecular biology type multi-step and multiplex reactions and analyses can be carried out on the device. The devices of this invention are able to electronically provide active or dynamic control over a number of important reaction parameters. This electronic control leads to significant improvements in reaction rates, specificities, and sensitivities. The improvements in these reaction parameters come from the ability of the device to electronically control and affect: (1) the rapid transport of reactants or analytes to a specific micro-location containing attached specific binding entities; (2) improvement in reaction rates due to the concentrated reactants or analytes reacting with the specific binding entities at that specific micro-location; and (3) the rapid and selective removal of un-reacted and non-specifically bound components from that micro-location. These advantages are utilized in a novel process called "electronic stringency control".

The self-addressed devices of this invention are able to rapidly carry out a variety of micro-formatted multi-step and/or multiplex reactions and procedures; which include, but are not limited to:

- DNA and RNA hybridizations procedures and analysis in conventional formats, and new improved matrix formats;
- molecular biology reaction procedures, e.g., restriction enzyme reactions and analysis, ligase reactions, kinasing reactions, and amplification procedures;

- antibody/antigen reaction procedures involving large or small antigens and haptens;
- diagnostic assays, e.g., hybridization analysis, gene analysis, fingerprinting, and immunodiagnostics;
- biomolecular conjugation procedures (i.e. the covalent and non-covalent labeling of nucleic acids, enzymes, proteins, or antibodies with reporter groups);
- biopolymer synthesis procedures, e.g., combinatorial synthesis of oligonucleotides or peptides;
- water soluble synthetic polymer synthesis, e.g., carbohydrates or linear polyacrylates; and
- macromolecular and nanostructure (nanometer size particles and structures) synthesis and fabrication.

NUCLEIC ACID HYBRIDIZATION

Nucleic acid hybridizations are used as examples of this invention because they characterize the most difficult multi-step and multiplex reactions.

The claimed device and methods allow nucleic acid hybridization to be carried out in a variety of conventional and new formats. The ability of the device to electronically control reaction parameters greatly improves nucleic acid hybridization analysis, particularly the ability of the device to provide electronic stringency control (ESC).

By "nucleic acid hybridization" is meant hybridization between all natural and synthetic forms and derivatives of nucleic acids, including: deoxyribonucleic acid (DNA), ribonucleic acid (RNA), polynucleotides and oligonucleotides.

Conventional hybridization formats, such as "dot blot" hybridization and "sandwich" hybridization, can be

carried out with the claimed device as well as large scale array or matrix formats.

As an example, a device for DNA hybridization analysis is designed, fabricated, and used in the following manner. Arrays of micro-locations are first fabricated using microlithographic techniques. The number of addressable micro-locations on an array depends on the final use. The device is rapidly self-addressed in a serial manner with a group of specific oligonucleotides. In this case, the specific oligonucleotides are 3'-terminal aldehyde functionalized oligonucleotides (in the range of 6-mer to 100-mer). The aldehyde functional group allows for covalent attachment to the specific micro-location attachment surface (see Figure 4). This group of specific oligonucleotides can be readily synthesized on a conventional DNA synthesizer using conventional techniques.

The synthesis of each specific oligonucleotide is initiated from a ribonucleotide controlled pore glass (CPG) support. Thus, the 3'-terminal position contains a ribonucleotide, which is then easily converted after synthesis and purification to a terminal dialdehyde derivative by periodate oxidation. The aldehyde containing oligonucleotides (40) will react readily with the primary amine functional groups on the surface of micro-locations by a Schiff's base reaction process.

The electronic addressing of the device with specific oligonucleotides is shown in Figure 8. The addressing of the first specific micro-location (ML-1) (81) with its specific sequence oligonucleotide (SSO-1) (82) is accomplished by maintaining the specific microelectrode (ML-1) at a positive DC potential, while all other microelectrodes are maintained at a negative potential (Fig. 8(A)). The aldehyde functionalized specific sequence (SSO-1) in aqueous buffered solution is free field electrophoresed to the ML-1 address, where it concentrates (> 10^6 fold) and

immediately becomes covalently bound to the surface of ML-1 (81). All other microelectrodes are maintained negative, and remain protected or shielded from reacting with SSO-1 sequence (82). The ML-1 potential is then reversed to negative (-) to electrophores any unreacted SSO-1 to a disposal system. The cycle is repeated, SSO-2 (84) ---> ML-2 (83), SSO-3 (86) ---> ML-3 (85), SSO-n ---> ML-n until all the desired micro-locations are addressed with their specific DNA sequences (Fig. 8(D)).

Another method for addressing the device is to transport specific binding entities such as specific oligonucleotides from an electronic reagent supply device. This supply device would hold a large quantity of binding entities or reagents and would be used to load analytical devices. Binding entities would be electronically transported between the two devices. Such a process eliminates the need for physical manipulations, such as pipetting, in addressing a device with binding entities.

Yet another method for addressing the device is to carry out the combinatorial synthesis of the specific oligonucleotides at the specific micro-locations. Combinatorial synthesis is described in a later section.

After the device is addressed with specific DNA sequences, the micro-locations on the array device remain as independent working direct current (DC) electrodes. This is possible because the attachment to the electrode surface is carried out in such a manner that the underlying micro-electrode does not become chemically or physically insulated. Each micro-electrode can still produce the strong direct currents necessary for the free field electrophoretic transport of other charged DNA molecules to and from the micro-location surface. The DNA array device provides complete electronic control over all aspects of the DNA hybridization and any other subsequent reactions.

An example of an electronically controlled hybridization process is shown in Figure 9. In this case, each addressable micro-location has a specific capture sequence (90). A sample solution containing target DNA (92) is applied to the device. All the micro-locations are activated and the sample DNA is concentrated at the micro-locations (Fig. 9(B)). Target DNA molecules from the dilute solution become highly concentrated at the micro-locations, allowing very rapid hybridization to the specific complementary DNA sequences on the surface. Reversal of the micro-electrode potential repels all un-hybridized DNA from the micro-locations, while the target DNA remains hybridized (Fig. 9(C)). In similar fashion, reporter probes are hybridized in subsequent steps to detect hybridized complexes.

The electronic control of the hybridization process significantly improves the subsequent detection of the target DNA molecules by enhancing the overall hybridization efficiency and by removing non-specific DNA from the micro-location areas. It is expected that 10,000 to 100,000 copies of target sequences in un-amplified genomic DNA will be detectable. Hybridization reactions of this type can be carried out in a matter of minutes, with minimal outside manipulations. Extensive washing is not necessary.

Another common format for DNA hybridization assays involves having target DNAs immobilized on a surface, and then hybridizing specific probes to these target DNAs. This format can involve either the same target DNAs at multiple locations, or different target DNAs at specific locations. Figure 10 shows an improved version of this serial hybridization format. In this case micro-locations (101-107) are addressed with different capture DNAs. These are hybridized in a serial fashion with different sequence specific oligonucleotides (108,109). The micro-locations are sequentially biased positive to transport

molecules to itself and then biased negative to transport molecules to the next micro-location. Specifically hybridized DNA will remain at the micro-location regardless of electrode potential. The sequence specific oligonucleotides can be labeled with a suitable reporter group such as a fluorophore.

The claimed device is able to provide electronic stringency control. Stringency control is necessary for hybridization specificity, and is particularly important for resolving one base mismatches in point mutations. Figure 11 shows how electronic stringency control can be used for improving hybridization specificity for one base mismatch analysis. The electronic stringency control can also be applied to multiple-base mismatch analysis.

Perfectly matched DNA hybrids (110) are more stable than mismatched DNA (112) hybrids. By biasing the micro-locations negative (Fig. 11(B)) and delivering a defined amount of power in a given time, it is possible to denature or remove the mismatched DNA hybrids while retaining the perfectly matched DNA hybrids (Fig. 11(C)). In a further refinement, the claimed device provides independent stringency control to each specific hybridization reaction occurring on the device. With a conventional or passive array format, it is impossible to achieve optimal stringency for all the hybridization events which are occurring in the same hybridization solution. However, the active array devices of this invention are able to provide different electronic stringency to hybridizations at different micro-locations, even though they are occurring in the same bulk hybridization solution. This attribute overcomes a major limitation to conventional matrix hybridization formats, sequencing by hybridization (SBH) formats, and other multiplex analyses.

The ability to provide electronic stringency control to hybridizations also provides mechanisms for detecting DNA hybridization without reporter group labeled DNA

probe. It provides a way to carry out a more direct detection of the hybridization process itself. A fluorescent dye detection process is shown in Figure 12 and described in Examples 4 and 6. Direct detection of DNA hybrids can be achieved by using DNA binding dyes such as ethidium bromide. The dye binds to both double-stranded and single-stranded DNA but with a greater affinity for the former. In Figure 12(B) positively charged dye (122) is transported to negatively biased micro-locations. The dye binds to both hybridized (120) and unhybridized (121) DNA sequences (Fig. 12c). By biasing the micro-locations positive and delivering a defined amount of power in a given amount of time, the dye molecules bound to unhybridized micro-locations is selectively removed. The amount of power applied does not adversely affect the DNA hybrids.

The hybridized DNAs with associated dye molecules are then fluorescently detected using associated or integrated optical systems.

The following reiterates the important advantages the devices of this invention provide for nucleic acid hybridization reactions and analysis:

(1) The rapid transport of dilute target DNA and/or probe DNA sequences to specific micro-location(s) where hybridization is to occur. This process takes place in no more than a few seconds.

(2) Concentrating dilute target DNA and/or probe DNA sequences at specific micro-location(s) where hybridization is to occur. The concentrating effect can be well over a million fold ($> 10^6$).

(3) The rapid removal of non-specifically bound target DNA sequences from specific micro-location(s) where hybridization has occurred. This process takes 10 to 20 seconds.

(4) Rapid removal of competing complementary target DNA sequences from specific micro-location(s) where hybridization has occurred. This process takes 10 to 20 seconds.

(6) The ability to carry out a complete hybridization process in several minutes.

(7) The ability to carry out a hybridization process with minimal outside manipulations or washing steps.

(8) The use of electronic stringency control (ESC) to remove partially hybridized DNA sequences.

(9) The ability to carry out hybridization analysis of un-amplified genomic target DNA sequences in the 1000 to 100,000 copy range.

(10) The use of ESC to improve the resolution of single base mis-match hybridizations (point mutations).

(11) The use of ESC to provide individual stringency control in matrix hybridizations.

(12) Improving the detection of hybridization event by removing non-specific background components.

(13) The development of new procedures which eliminate the need for using covalently labeled reporter probes or target DNA to detect the hybridization events.

REPRODUCTION OF DEVICES

In addition to separately addressing individual devices with specific binding entities, it is also possible to produce a master device, which can copy specific binding entities to other devices. This represents another method for the production of devices. The process for the replication of devices is shown in Figure 13. A master device containing micro-locations which have been addressed with specific binding sequences is hybridized with respective complementary DNA sequences (130). These

complementary sequences are activated and thus capable of covalent binding to the micro-location attachment layer.

An unaddressed sister device (132) containing an attachment layer is aligned with the hybridized master device (Fig. 13(B)). The master device micro-locations are biased negative and the sister device micro-locations are biased positive. The DNA hybrids are denatured and are transported to the sister device, where the activated DNA sequence binds covalently to the micro-location (Fig. 13(C)). The process can be performed in parallel or in series, depending on the device geometry so that crosstalk between the micro-locations is minimized. The hybrids can be denatured by applying a sufficient negative potential or by using a positively charged chaotropic agent or denaturant.

DETECTION SYSTEM

In the case of fluorescent binding reactions, it is possible to use an epifluorescent type microscopic detection system for the analysis of the binding reactions. The sensitivity of the system depends on the associated imaging detector element (CCD, ICCD, Microchannel Plate) or photon counting PMT system. One alternative is to associate a sensitive CCD detector or avalanche photodiode (APD) detector directly with the device in a sandwich arrangement. Another alternative is to integrate opto-electronic or microelectronics detection in the device.

COMBINATORIAL BIOPOLYMER SYNTHESIS

The devices of this invention are also capable of carrying out combinatorial synthesis of biopolymers such as oligonucleotides and peptides. Such a process allows self-directed synthesis to occur without the need for any outside direction or influence. This combinatorial synthesis allows very large numbers of sequences to be synthesized on a device. The basic concept for combina-

torial synthesis involves the use of the device to transport, concentrate, and react monomers, coupling reagents, or deblocking reagents at the addressable micro-locations. The concept capitalizes on the ability of the device to protect certain locations from the effects of nearby reagents. Also important to the concept is the identification of selective steps in these chemical synthesis processes where one or more of the reactants has either a net positive or negative charge, or to create such suitable reagents for these processes.

One method for combinatorial oligonucleotide synthesis is shown in Figure 14. This method begins with a set of selectively addressable micro-locations (140) whose surfaces have been derivatized with blocked primary amine (X-NH-) groups (142). The initial step in the process involves selective deblocking of electrodes using a charged deblocking reagent (144). In this case, the reagent would carry a positive (+) charge. The process is carried out by applying a negative potential to those electrodes being de-blocked, and a positive potential to those which are to remain protected (Fig. 14(B)). Application of positive and negative potentials to selective electrodes causes the charged reagents to be concentrated at those micro-locations being de-blocked, and excludes the reagents from the other electrode surfaces.

In the second step, chemical coupling of the first base, in this case cytosine, to the deblocked micro-locations is carried out by simply exposing the system to the phosphoramidite reagent (x-C) (146). The (C) nucleotide couples to de-blocked micro-location surfaces, but not to any of the blocked electrode surfaces (Fig. 14(C) and (D)). At this point normal phosphoramide chemistry IS carried out until the next de-blocking step.

At the second de-blocking step (Fig. 14(D)), those electrode positions which are to be coupled with the next

base are made negative, and those which are to remain protected are made positive. The system is now exposed to the next base to be coupled, in this case (x-A) (148), and selective coupling to the de-blocked micro-location is achieved (Fig. 14(E) and (F)). The coupling and de-blocking procedures are repeated, until all the different DNA sequences have been synthesized on each of the addressable micro-location surfaces.

The above example represents one possible approach for the synthesis of nucleic acids. Another approach involves a complete water soluble DNA synthesis. In this case, charged water soluble coupling agents, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCA), is used to carry out oligonucleotide synthesis with water soluble nucleotide derivatives. This approach would have significant advantages over present organic solvent based methods which require extensive blocking of the base moieties. Water soluble synthesis would be less expensive and eliminate the use of many toxic substances used in the present organic solvent based processes. A third approach involves the use of charged monomers.

In addition to DNA synthesis, a similar process can be developed for peptide synthesis, and other complex polymers. Examples contemplated in this disclosure represent the initial potential for this technique, and are based on organic solvent based synthetic procedures for DNA or peptide synthesis.

The recipes for buffers, solutions, and media in the following examples are described in J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

Example 1: Oligomer Reagents

Synthetic DNA probes were made using conventional phosphoramidite chemistry on Applied Biosystems automated

DNA synthesizers. Oligomers were designed to contain either a 5' amino or a 3' ribonucleoside terminus. The 5' functionality was incorporated by using the ABI Aminolink 2 reagent and the 3' functionality was introduced by initiating synthesis from an RNA CPG support. The 3' ribonucleotide terminus can be converted to a terminal dialdehyde by the periodate oxidation method which can react with primary amines to form a Schiff's base. Reaction conditions were as follows: Dissolve 20-30 O.D. oligomer in water to a final concentration of 1 OD/μl. Add 1 vol of 0.1M sodium acetate, pH 5.2 and 1 vol 0.45M sodium periodate (made fresh in water). Stir and incubate reaction for at least 2 hours at ambient temperature, in the dark. Load reaction mix onto a Sephadex G-10 column (pasteur pipette, 0.6 X 5.5 cm) equilibrated in 0.1M sodium phosphate, pH 7.4. Collect 200 μl fractions, spot 2 μl aliquots on thin layer chromatography (TLC) and pool ultra violet (UV) absorbing fractions.

The following oligomers contain 3' ribonucleoside termini (U):

ET12R	5'- GCT AGC CCC TGC TCA TGA GTC TCU
CP-1	5'- AAA AAA AAA AAA AAA AAA AAU
AT-A1	5'- CTA CGT GGA CCT GGA GAG GAA GGA GAC TGC CTG U
AT-A2	5'- GAG TTC AGC AAA TTT GGA GU
AT-A3	5'- CGT AGA ACT CCT CAT CTC CU
AT-A4	5'- GTC TCC TTC CTC TCC AGU
AT-A5	5'- GAT GAG CAG TTC TAC GTG GU
AT-A6	5'- CTG GAG AAG AAG GAG ACU
AT-A7	5'- TTC CAC AGA CTT AGA TTT GAC U
AT-A8	5'- TTC CGC AGA TTT AGA AGA TU
AT-A9	5'- TGT TTG CCT GTT CTC AGA CU
AT-A10	5'- CAT CGC TGT GAC AAA ACA TU

Oligomers containing 5' amine groups were generally reacted with fluorophores, such as Texas Red (TR, ex. 590nm, em. 610nm). Sulfonyl chlorides are very reactive towards primary amines forming a stable sulfonamide

linkage. Texas Red-DNA conjugates were made as follows: Texas Red sulfonyl chloride (Molecular Probes) was dissolved in dimethyl formamide (DMF) to a final concentration of 50 mg/ml (80 mM). Oligomer was dissolved in 0.4M sodium bicarbonate, pH 9.0-9.1, to a final concentration of 1 O.D./μl (5.4 mM for a 21-mer). In a micro test tube, 10 μl oligomer and 20 μl Texas Red was combined. Let reaction proceed in the dark for 1 hour. Quench reaction with ammonia or hydroxylamine, lyophilize sample and purify by PAGE (Sambrook et al., 1989, supra).

The following oligomers contain 5' amino termini:

ET21A 5'- Aminolink2 - TGC GAG CTG CAG TCA GAC AT
ET10AL 5'- Aminolink2 - GAG AGA CTC ATG AGC AGG
ET11AL 5'- Aminolink2 - CCT GCT CAT GAG TCT CTC
T2 5'- Aminolink2 - TTT TTT TTT TTT TTT TTT TT
RC-A1 5'- Aminolink2 - CAG GCA GTC TCC TTC CTC TCC AGG TCC ACG TAG
RC-A2 5'- Aminolink2 - CTC CAA ATT TGC TGA ACT C
RC-A3 5'- Aminolink2 - GGA GAT GAG GAG TTC TAC G
RC-A4 5'- Aminolink2 - CTG GAG AGG AAG GAG AC
RC-A5 5'- Aminolink2 - CCA CGT AGA ACT GCT CAT C
RC-A6 5'- Aminolink2 - GTC TCC TTC TTC TCC AG
RC-A7 5'- Aminolink2 - GTC AAA TCT AAG TCT GTG GAA
RC-A8 5'- Aminolink2 - ATC TTC TAA ATC TGC GGA A
RC-A9 5'- Aminolink2 - GTC TGA GAA CAG GCA AAC A
RC-A10 5'- Aminolink2 - ATG TTT TGT CAC AGC GAT G

Example 2: <u>Electronically Addressable Micro-locations on a Microfabricated Device - Polylysine Method</u>

Microelectrodes were fabricated from microcapillary tubes (0.2 mm x 5 mm). The microcapillaries were filled with 18-26% polyacrylamide containing 0.1 - 1.0% poly-lysine and allowed to polymerize. The excess capillary was scored and removed to prevent air bubbles from being trapped within the tubes and to standardize the tube

length. Capillaries were mounted in a manner such that they shared a common upper buffer reservoir and had individual lower buffer reservoirs. Each lower buffer reservoir contained a platinum wire electrode.

The top surface of the microcapillary in the upper reservoir was considered to be the addressable micro-location. Upper and lower reservoirs were filled with 0.1 M sodium phosphate, pH 7.4 and prerun for 10' at 0.05 mA constant using a BioRad 500/1000 power supply. Pipette 2 μl (0.1 O.D.) periodate oxidized ET12R into the upper reservoir while the power is on and electrophorese for 2-5 minutes at constant current. Reverse polarity so that the test capillary is now biased negative and electrophorese an additional 2-5 minutes. Unbound DNA is repulsed while the covalently attached DNA remains.

Aspirate upper reservoir buffer and rinse with buffer. Disassemble apparatus and mount a fresh reference capillary. Refill reservoir and add fluorescently labeled complement DNA, i.e., ET10AL-TR. Electrophoretically concentrate the oligomer at the positively biased test micro-location for 2-5 minutes at 0.05 mA constant. Reverse the polarity and remove unbound complement. Remove test capillary and examine by fluorescence. Negative control for nonspecific binding was performed as described above substituting a noncomplementary DNA sequence ET21A-TR for ET10AL-TR.

A cross-section of the capillary micro-locations were examined under a Jena epifluorescent microscope fitted with a Hamamatsu ICCD camera imaging system. Fluorescent results indicate that complement ET10AL-TR hybridized to the binding entity/capture sequence and remained hybridized even when the potential was changed to negative. ET21A-TR noncomplement was not retained at the test capillary when the potential was reversed.

Example 3: Electronically Addressable Micro-locations on a Microfabricated Device - Succinimidyl Acrylate Method

This example describes an alternative attachment chemistry which covalently binds the 5' terminus of the oligomer. Capillaries were fabricated as described above except that 1% succinimidyl acrylate (Molecular Probes) was substitute for the polylysine. The capillaries were made fresh because the succinimidyl ester reacts with primary amines and is labile, especially above pH 8.0. The capillaries were mounted as described above and the reservoirs were filled with 0.1 M sodium phosphate, pH 7.4. Prerun the capillaries for 10 minutes at 0.05 mA. Pipette 2 µl ET10AL (0.1 O.D.), which contains a 5' amino terminus, into the upper reservoir while the power is on and electrophorese for 2-5 minutes. Reverse polarity so that the test capillary is now biased negative and electrophorese an additional 2-5 minutes. Unbound DNA is repulsed while the covalently attached DNA remains.

Aspirate upper reservoir buffer and rinse with buffer. Unmount the reference capillary and mount a fresh reference capillary. Refill reservoir and add fluorescent labeled complement oligomer, ET11AL-TR and electrophorese as described above. Negative control for nonspecific binding was performed as described above substituting a noncomplement DNA sequence ET21A-TR for ET11AL-TR.

Fluorescent results indicate that complement ET11AL-TR hybridized to the capture sequence and remained hybridized even when the potential was changed to negative. ET21A-TR noncomplement was not retained at the working capillary when the potential was reversed.

Example 4: Electronically Controlled Fluorescent Dye Detection Process-PAGE

DNA dyes such as ethidium bromide (EB) become fluorescent when intercalated into DNA. The fluorescence and

binding affinity is greater when the DNA is double stranded than single stranded. Prepare capillaries as in Example 1 and hybridize as described above. EB was added to the solution (~ 0.05 mM EB final concentration) and the test capillary was biased negative because EB is positively charged. The capillaries were observed by epifluorescence at 550 nm excitation and 600+ nm emission. Both the hybridized and unhybridized micro-locations showed red fluorescence (from EB).

The capillaries were re-mounted biased positive to repulse EB. Maintain constant current at 0.05 mA for 0.03 Volt-Hours.

Capture	Target	Normalized Signal
ET10AL	ET11AL (Pos.)	>200
ET10AL	ET21A (Neg.)	1

Fluorescence at the unhybridized micro-locations diminished while the hybridized capillary retained fluorescence. Fluorescent signal was measured using an ICCD camera imaging system and represent peak fluorescent intensities. The signal to noise ratio would be >>1000 fold if the entire fluorescent signal area was integrated. This demonstrates a method for increasing signal to noise ratios and thus the dynamic range of the assay.

Example 5: <u>Electronically Addressable Locations on Metal Substrates</u>

Aluminum (Al) and gold (Au) wire (0.25 mm, Aldrich) was reacted with 10% 3-aminopropyltriethoxysilane (APS) in toluene. The APS reagent reacts readily with the oxide and/or hydroxyl groups on the metal surface to form covalent bonds between the oxide and/or hydroxyl groups and the primary amine groups. No pretreatment of the aluminum was necessary. The gold wire was subjected to electrolysis in 5 x SSC solution to form an oxide layer. Alternatively the metal wire can be oxidized by a perchloric acid bath.

The APS reaction was performed as follows: Wires were cut to 3 inches and placed in a glass dish. Toluene was added to completely cover the wires and the temperature was brought to 50-60 °C on a heat plate. APS was added to a final concentration of 10%. Mix solution and continue the reaction for 30 minutes. Rinse 3 times with copious volumes of toluene, then rinse 3 times with copious volumes of alcohol and dry in 50°C oven. The APS treated wire can then be reacted with an aldehyde to form a Schiff's base. Binding entity ET12R was periodate oxidized as described elsewhere. The electrodes were placed in a reservoir of degassed water. Power was applied at .05 mA constant for about 30 seconds. Activated ET12R was immediately added. Power was applied, the liquid was aspirated and fresh water was added and then aspirated again. The test (biased positive) and reference electrodes were placed in Hybridization Buffer (HB, 5XSSC, 0.1% SDS) containing fluorescent labeled complement DNA, ET10-TR. After 2 minutes the electrodes were washed three times for one minute each in Wash Buffer (1 x SSC, 0.1% SDS) and observed by fluorescence (ex. 590 nm, em. 610 nm).

Results demonstrate that ET12R was specifically coupled to the treated metal surfaces. The test electrode was fluorescent while the reference electrode was not. Nonspecific adsorption of the DNA to the metal was prevented by the presence of SDS in the Hybridization Buffer. Attachment to gold substrates by electrolysis and subsequent APS treatment was effective. Signal obtained was significantly stronger than observed with non-oxidized gold. More importantly, this example showed that the metal surfaces could be chemically functionalized and derivatized with a binding entity and not become insulated from the solution. The APS method represents one of many available chemistries to form DNA-metal conjugates.

Example 6: Electronically Controlled Fluorescent Dye Detection Process - Metal Wire

DNA-aluminum electrode substrates were prepared and hybridized as described in Example 5. A hybridized and an unhybridized DNA-Al electrode were processed with an underivatized Al wire as the reference. EB was added to the solution and the test DNA electrodes were biased negative to attract the dye. The solution was aspirated and fresh buffer was added. The metal surfaces were examined under the microscope.

Remount the device and apply a positive potential for a defined volt-hour. The buffer was aspirated, the electrodes were observed by epifluorescence. This was repeated until there was a significant difference in fluorescence between the hybridized and unhybridized metal surfaces.

Capture	Target	Normalized Signal
ET12R	ET10AL (Pos.)	>140
ET12R	None (Neg.)	1

Fluorescence at the unhybridized metal surfaces diminished while the hybridized metal surfaces retained fluorescence. Fluorescent signal was measured using an ICCD camera imaging system and represent peak fluorescent intensities. The signal to noise ratio would be >>1000 fold if the entire fluorescent signal area was integrated. This example demonstrates a method for increasing signal to noise ratios and thus the dynamic range of the assay. Similar results were obtained using capillary gel configuration, suggesting that electrochemical effects do not significantly affect the performance of the assay.

Example 7: Active Programmable Electronic Matrix (APEX) - Micro-machine Fabrication

A radial array of 6 addressable 250 μm capillary locations was micro-machined. The device has a common upper reservoir and separate lower reservoirs such that

the potential at each micro-location is individually addressable. A unique oligomer binding entity is localized and coupled to a specific capillary micro-location by the methods described elsewhere. The test micro-location has a positive potential while the other micro-locations have negative potentials to prevent nonspecific interactions.

The array is washed and then hybridized with a complementary fluorescently labeled DNA probe. The array is washed to remove excess probe and then observed under an epifluorescent microscope. Only the specifically addressed micro-location will be fluorescent. The process will be repeated with another binding entity at another location and verified by hybridization with a probe labeled with another fluorescent moiety.

DNA sequences are specifically located to predetermined positions with negligible crosstalk with the other locations. This enables the fabrication of micromatrices with several to hundreds of unique sequences at predetermined locales.

Example 8: Active, Programmable Electronic Matrix (APEX) - Microlithographic Fabrication

An 8 X 8 matrix of 50 μm square aluminum electrode pads on a silicon wafer (see Figure 3) was designed, fabricated and packaged with a switch box (see Device Fabrication Section for details). Several materials and process improvements, as described below, were made to increase the selectivity and effectiveness of the chip.

8a) Selection of Topcoat

The APS process involves the entire chip. Selectivity of the functionalization process was dependent on the reactivity of the chip surfaces. In order to reduce functionalization and subsequent DNA attachment of the areas surrounding the micro-locations, a material that is less reactive to APS than SiO_2 or metal oxide is needed. Photo-

resists and silicon nitride were tried. The different topcoats were applied to silicon dioxide chips. The chips were examined by epifluorescence and the then treated with APS followed by covalent attachment of periodate oxidized polyA RNA sequences (Sigma, MW 100,000). The chips were hybridized with 200 nM solution of Texas Red labeled 20-mer (T2-TR) in Hybridization Buffer, for 5 minutes at 37°C. The chips were washed 3 times in WB and once in 1 x SSC. The chips were examined by fluorescence at 590 nm excitation and 610 nm emission.

Silicon nitride was chosen because it had much less reactivity to APS relative to silicon dioxide and was not inherently fluorescent like the photoresist tested. Other methods such as UV burnout of the background areas are also possible.

8b) APEX Physical Characterization

A finished matrix chip was visually examined using a Probe Test Station (Micromanipulator Model 6000) fitted with a B & L microscope and a CCD camera. The chip was tested for continuity between the test pads and the outer contact pads. This was done by contacting the pads with the manipulator probe tips which were connected to a multimeter. Continuity ensures that the pads have been etched down to the metal surface. The pads were then checked for stability in electrolytic environments. The metal wires were rated to handle up to 1 mA under normal dry conditions. However, reaction to a wet environment was unknown. A drop (1-5 μl) of buffered solution (1 x SSC) was pipetted onto the 8X8 matrix. Surface tension keeps the liquid in place leaving the outer contact pad area dry. A probe tip was contacted to a contact pad and another probe tip was contacted with the liquid. The current was incremented up to 50 nA at max voltage of 50 V using a HP 6625A power supply and HP3458A digital multimeter.

The initial fabrication consisted of the silicon substrate, a silica dioxide insulating layer, aluminum deposition and patterning, and a silicon nitride topcoat. These chips were not very stable in wet environments because the metal/nitride interface was physical in nature and electrolysis would undermine the nitride layer. This would result in the pads being electrically shorted. Furthermore, silicon nitride and Al have different expansion coefficients such that the nitride layer would crack when current was applied. This would allow solution to contact the metal directly, again resulting in electrolysis which would further undermine the layer.

The second fabrication process included a silicon dioxide insulating layer between the aluminum metal and silicon nitride layers. Silicon dioxide and Al have more compatible physical properties and form a better chemical interface to provide a more stabile and robust chip.

8c) DNA Attachment

A matrix chip was functionalized with APS reagent as described in Example 5. The chip was then treated with periodate oxidized polyA RNA (Sigma, average MW 100,000). The chip was washed in WB to remove excess and unbound RNA. This process coated the entire chip with the capture sequence with a higher density at the exposed metal surfaces than at the nitride covered areas. The chip was hybridized with a 200 nM solution of T2-TR in HB for 5 minutes at 37°C. Then washed 3 times in WB and once in 1XSSC for one minute each at ambient temperature. The chip was examined by fluorescence at 590 nm excitation and 610 nm emission.

The opened metal areas were brightly fluorescent and had the shape of the pads. Low fluorescent intensities and/or irregular borders would suggest that the pads were not completely opened. Additional plasma etch times would be recommended.

8d) Electronically Controlled Hybridization

Active hybridization was performed by using a chip from Example 8c and biasing one micro-location positive. This was done by using the switch box which would also automatically bias the remaining micro-locations negative or by using an external solution electrode. Three microliters of water was deposited on the matrix pads only. A current, ~1-5 nA, was applied for several seconds and 0.1 pmole of T2-TR was added to the solution. The liquid was removed and the chip was dried and examined by fluorescence at Texas Red wavelengths (ex.590 nm, em.610 nm).

Only the positively biased micro-location was fluorescent. This can be repeated many times to hybridize other micro-locations selectively. Additionally, the fluorescence DNA at one micro-location can be translocated to another micro-location by biasing the initial location negative and the destination positive.

8e) Electronically Controlled Addressing and Device Fabrication

The matrix was functionalized with APS as described above. Binding entity CP-1 was activated by periodate oxidation method. Four micro-locations were biased positive in the matrix and the remainder were biased negative. Two microliters of water was deposited on the matrix and a current was applied. Binding entity, CP-1, was added and allowed to concentrate at the designated locations. The liquid was removed, the chip was rinsed briefly with water and two microliters of water was deposited on the chip. Again, current was applied for several seconds and 0.1 pmole of T2-TR was added. The liquid was removed after a short time and the entire chip was washed in WB, 3 times. The chip was dried and examined for fluorescence.

Results indicate that the positively biased micro-locations were fluorescent. This example demonstrates the selective addressing of micro-locations with a specific binding entity, the localization and covalent coupling of sequences to the micro-locations, and the specific hybridization of complementary target sequences to the derivatized micro-locations.

8f) Genetic Typing APEX Chip

DNA binding entities with 3' ribonucleoside termini are synthesized which are specific for the polymorphisms of HLA gene dQa. The binding entities are activated by periodate oxidation as described above. The reverse complements are also synthesized with 5' amino termini and are conjugated with fluorophores, such as Texas Red, Rhodamine or Bodipy dyes, as described elsewhere. The micro-locations are functionalized with primary amines by treatment with APS, as described elsewhere. A couple microliters of solution are placed over the matrix but leaving the contact pads dry. A specific micro-location is addressed by biasing that micro-location positive, the periodate oxidized DNA oligomer is added, ~0.1 pmole, and is translocated and covalently coupled to that location. The polarity is reversed and the unbound binding entity molecules are removed. This is repeated for another binding entity at another addressed micro-location until all the unique binding entities are bound to the chip. The chip is then hybridized to individual fluorescently labeled complement sequences to determine the specificity of the coupling reaction as well as en masse to visualize all addressed micro-locations at once. On the same chip which is denatured to remove complementary oligomers (10 minutes at 90°C in 0.05% SDS), the addressed micro-locations are hybridized with unlabeled reverse complements or genomic DNA. Detection is via the fluorescent dye detection assay as described elsewhere.

Results will demonstrate that micro-locations are specifically addressed with unique binding entities. Nonspecific binding to negatively biased micro-locations will be negligible. The device and associated binding entity chemistry is stable under denaturation conditions, thus making the addressed and fabricated device reusable. Alternative methods for denaturing the hybrids would be to increase the current and/or increase the time it is applied.

Example 9: Electronic Stringency Control

The ability of the device to affect electronic stringency control is demonstrated with the Ras oncogene model system. A single base pair mismatch adversely affects the melting temperature (Tm), a measure of the stability of the duplex. Traditional methods to discriminate between mismatch and perfect match (i.e., stringency control) rely on temperature and salt conditions. Stringency can also be affected by the electrophoretic potential. Oligomers listed below can be paired such that resulting hybrids have 0-2 mismatches. Oligomer binding entities are coupled to the micro-location and hybridized as described elsewhere. The polarity at the micro-location is then reversed and the hybrids are subjected to constant current for a given time, or defined power levels to denature the mismatch without affecting the perfect match.

Ras-G 5'- GGT GGT GGG CGC CGG CGG TGT GGG CAA GAU -3'
Ras-1 3'- CC GCG GCC GCC ACA C - Aminolink2 -5'
Ras-2 3'- CC GCG GCA GCC ACA C - Aminolink2 -5'
Ras-3 3'- CC GTG GCA GCC ACA C - Aminolink2 -5'
Ras-T 5'- GGT GGT GGG CGC CGT CGG TGT GGG CAA GAU -3'

Microelectrodes are fabricated from microcapillary tubes as described elsewhere. Binding entities Ras-G is periodate oxidized and covalently bound to the addressed micro-location. Ras-G micro-location is then hybridized

with Ras-1-TR which is the perfect match, Ras-2-TR which is a one base pair mismatch (G-A) or Ras-3-TR which is a two base pair mismatch (G-A and G-T). The micro-locations are examined fluorescently to verify whether complementary sequences are hybridized and to what extent. The micro-capillaries are re-mounted and subjected to controlled time at constant current until the mismatched hybrids are removed without significantly affecting the perfectly matched hybrids.

Results will indicate that stringency could be affected by the electrophoretic potential. This example demonstrates that each micro-location can have individual stringency control, thus overcomes a major obstacle to large scale parallel processing techniques which had been limited to a single common stringency level.

What is claimed is:

1. A self-addressable electronic device comprising:

a substrate,

a first selectively addressable electrode, the electrode being supported by the substrate,

a permeation layer, the permeation layer being disposed adjacent the first selectively addressable electrode,

a current source operatively connected to the first selectively addressable electrode, and

an attachment layer adjacent the permeation layer.

2. The electronic device of claim 1, further including a second selectively addressable electrode, the second electrode being supported by the substrate.

3. The electronic device of claim 1 or 2, further including an attachment layer, the attachment layer being disposed upon the permeation layer.

4. The electronic device of claim 1, wherein the substrate includes a base and an overlying insulator.

5. The electronic device of claim 1, wherein the substrate is chosen from the following group: silicon, glass, silicon dioxide, plastic, or ceramic materials.

6. The electronic device of claim 4, wherein the base is chosen from the following group: silicon, glass, silicon dioxide, plastic, or ceramic materials.

7. The electronic device of claim 4, wherein the base material is silicon.

8. The electronic device of claim 4, wherein the insulator is silicon dioxide.

9. The electronic device of claim 1, wherein the substrate comprises a circuit pattern or board.

10. The electronic device of claim 2, wherein the first selectively addressable electrode and the second selectively addressable electrode are separated by an insulator supported by the substrate.

11. The electronic device of claim 10, wherein the insulator is chosen from the following group: silicon dioxide, plastic, glass, resist, rubber, or ceramic materials.

12. The electronic device of claim 10, wherein silicon nitride is disposed upon the insulator.

13. The electronic device of claim 1, wherein the current source is a direct current source.

14. The electronic device of claim 1, wherein the permeation layer is aminopropyltriethoxy silane.

15. The electronic device of claim 1, wherein the permeation layer and the selectively addressable electrode are separated by a buffer reservoir.

16. The electronic device of claim 1, wherein the electrode is chosen from the following group: aluminum, gold, silver, tin, copper, platinum, palladium, carbon, semiconductor materials, and combinations thereof.

17. A self-addressable electronic device comprising:

a substrate,

a plurality of selectively addressable electrodes, the electrodes being disposed upon the substrate,

a current source,

electrical connections to the electrodes, the electrical connections providing a selective current path from the current source, and

a permeation layer adjacent each electrode, forming addressable binding locations.

18. The electronic device of claim 17, further comprising a switch controller for selectively connecting said current source to said addressable electrodes.

19. The electronic device of claim 17, further comprising an attachment layer disposed on said permeation layer, forming addressable binding locations.

20. The electronic device of claim 17, wherein the electrode material is chosen from the group: aluminum, gold, silver, tin, copper, platinum, palladium, carbon, semiconductor material, and combinations thereof.

21. The electronic device of claim 17, further including an electronic insulative material disposed between said plurality of selectively addressable electrodes.

22. The electronic device of claim 17, wherein the plurality of addressable binding locations are arranged in an array.

23. The electronic device of claims 17, further including a cavity for holding a solution including binding entities, reagents, and analytes.

24. The electronic device of claim 17, wherein specific binding entities have been selectively transported and bound to said addressable binding locations, forming an addressed active location device.

25. The electronic device of claim 17, wherein the width of the binding locations on the device is between 0.5 microns and 200 microns.

26. The electronic device of claim 17, wherein the width of the binding locations on the device is between 5 microns and 100 microns.

27. A self-addressable electronic device comprising:

a substrate,

a plurality of selectively addressable electrodes, the electrodes being disposed upon the substrate,

a current source,

electrical connections to the electrodes, the electrical connections providing a selective current path from the current source,

individual buffer reservoirs associated with said electrodes,

individual permeation layers disposed adjacent said individual buffer reservoirs, forming addressable binding locations.

28. The electronic device of claim 27, further comprising a common reservoir for containing solutions including binding entities, reagents, and analytes.

29. The electronic device of claim 27, further comprising an attachment layer disposed on said permeation layer, forming addressable binding locations.

30. The electronic device of claim 27, wherein said addressable binding locations are arranged in an array.

31. The electronic device of claim 27, wherein the permeation layer is selected from the group comprising: functionalized hydrophilic gels, membranes, and porous materials.

32. The electronic device of claim 27, wherein specific binding entities have been selectively transported and bound to said addressable binding locations, forming an addressed active location device.

33. The electronic device of claim 27, wherein the width of the locations on the device is between 50 microns and 2 centimeters.

34. The electronic device of claim 27, wherein the width of the locations on the device is between 100 microns and 5 millimeters.

35. A method for electronically controlling hybridization of DNA from a solution containing specific binding and non-specific binding DNA sequences to a binding location, comprising the steps of:

placing the solution in contact with a first binding location including a first underlying electrode, and a second binding location including a second underlying electrode;

placing said first binding location at a positive potential, relative to said second binding location, concentrating DNA on said first location surface; and

placing said first binding location at a negative potential, relative to said second binding location, wherein said negative potential or current is sufficient to remove the non-specifically bound DNA sequences from said first binding location, but not sufficient to remove the specifically bound DNA sequences.

36. A method for electronically controlling hybridization of DNA from a solution containing specific binding and non-specific binding DNA sequences to first and second binding locations, comprising the steps of:

placing the solution in contact with the first, second, and a third locations;

placing said first and second binding locations at a positive potential and said third location at a negative potential, concentrating DNA on said first and second locations;

placing said first and second specific binding locations at a negative potential and said third location at a positive potential; and

placing said first and second binding locations at negative potentials, relative to said third location, wherein said negative potential or current is sufficient to remove the non-specifically bound DNA from said first and second locations, but not sufficient to remove the specifically bound DNA sequences.

37. A method for electronically controlling hybridization of DNA from a solution containing

specific and non-specific DNA sequences to a first binding location and then to a second specific binding location, comprising the steps of:

placing the solution in contact with said first, second, and a third location;

placing said first binding location at a positive potential and said second binding location at a negative potential, concentrating DNA on said first location;

placing said first binding location at a negative potential and said second binding location at a positive potential, concentrating DNA on said second location; and

placing said first and second binding locations at negative potentials, relative to said third binding location, wherein said negative potential or current is sufficient to remove the non-specifically bound DNA from said first and second locations but not sufficient to remove the specifically bound DNA.

38. The method of hybridization of claim 37 wherein said negative potential or current is increased or decreased incrementally.

39. The method of claim 36 or 37 wherein multiple specific and non-specific DNA sequences are applied to an array of binding locations.

40. A method for actively transporting DNA from a solution to a plurality of locations, comprising the steps of:

placing a solution containing DNA in contact with a first, second, third, and n-number of locations;

providing a positive potential on said first location relative to other locations, transporting DNA to said first location;

providing a positive potential on said second location relatively to said first location, transporting DNA to said second location;

providing a positive potential to said third location relative to the second location, transporting DNA to said third location; and

repeating the process through n-number of locations.

41. An electronic controlled method for combinatorial synthesis of a biopolymer, comprising the steps of:

forming a plurality of reaction locations on a substrate, each reaction location being individually electronically addressable;

forming an attachment layer upon each reaction location;

placing said reaction locations in contact with a solution containing a charged monomer-A;

selectively biasing those locations at which reaction A is to occur at an opposite charge to monomer-A, and biasing those locations at which no reaction A is to occur the same charge as monomer-A;

concentrating and reacting monomer A on the specific A locations;

removing solution containing unreacted monomer A;

placing said reaction locations in contact with a solution containing a charged monomer B;

selectively biasing those locations for which reaction B is to occur at the opposite charge of monomer-B, and biasing those locations at which no reaction B is to occur the same charge as monomer-B;

concentrating and reacting monomer B on the specific B locations; and

repeating the process with monomer-A, monomer-B, to monomer-N, for n-number of times until all biopolymer sequences are complete.

42. A method for replicating a self-addressable electronic device addressed with specific DNA sequences, comprising the steps of:

hybridizing the complimentary sequences to the specific DNA sequences addressed on a master self-addressable electronic device;

aligning unaddressed locations on a recipient self-addressable electronic device with the addressed locations on said master device; and

biasing the locations on said master device negative and the locations on said recipient device positive, transporting the complimentary sequences to said recipient device.

43. The method for replicating patterned sequences of claim 42, further comprising denaturing the complimentary sequences from the master template.

44. A system for the detection of fluorescent or colorimetric binding reactions and assays, comprising:

two or more addressable locations; and

a detector system positioned adjacent to at least one of the locations.

45. The detection system of claim 44, wherein the detector is an optoelectronic detector chosen from the group: photodiode, avalanche photodiode, or photomultiplier tube.

46. The detection system of claim 44, wherein the detector is an optoelectronic imaging detector chosen from the group: charged coupled device, cooled charged coupled device, intensified charged coupled device, or microchannel device.

47. The detection system of claim 44, wherein the detector is capable of detecting the emission of fluorescent radiation.

48. The detection system of claim 44 wherein the detector is capable of detecting the absorption of spectrophotometric radiation.

AMENDED CLAIMS

[received by the International Bureau on 20 April 1995 (20.04.95); original claims 44-48 amended; new claim 49 added; remaining claims unchanged (2 pages)]

concentrating and reacting monomer B on the specific B locations; and

repeating the process with monomer-A, monomer-B, to monomer-N, for n-number of times until all biopolymer sequences are complete.

42. A method for replicating a self-addressable electronic device addressed with specific DNA sequences, comprising the steps of:

hybridizing the complimentary sequences to the specific DNA sequences addressed on a master self-addressable electronic device;

aligning unaddressed locations on a recipient self-addressable electronic device with the addressed locations on said master device; and

biasing the locations on said master device negative and the locations on said recipient device positive, transporting the complimentary sequences to said recipient device.

43. The method for replicating patterned sequences of claim 42, further comprising denaturing the complimentary sequences from the master template.

44. The self-addressable electronic device of claim 1 further including a system for the detection of fluorescent or colorimetric binding reactions and assays, comprising:

a detector system positioned adjacent the selectively addressable electrode.

45. The detection system of claim 44 or 49, wherein the detector is an optoelectronic detector chosen from the group: photodiode, avalanche photodiode, or photomultiplier tube.

46. The detection system of claim 44 or 49, wherein the detector is an optoelectronic imaging detector chosen from the group: charged coupled device, cooled charged coupled device, intensified charged coupled device, or microchannel device.

47. The detection system of claim 44 or 49, wherein the detector is capable of detecting the emission of fluorescent radiation.

48. The detection system of claim 44 or 49 wherein the detector is capable of detecting the absorption of spectrophotometric radiation.

49. A system for the detection of fluorescent or colorimetric binding reactions and assays, comprising:

two or more addressable locations on a substrate; and

one or more detector systems positioned adjacent to at least one of the locations and formed integral with the substrate.

FIG. 1.

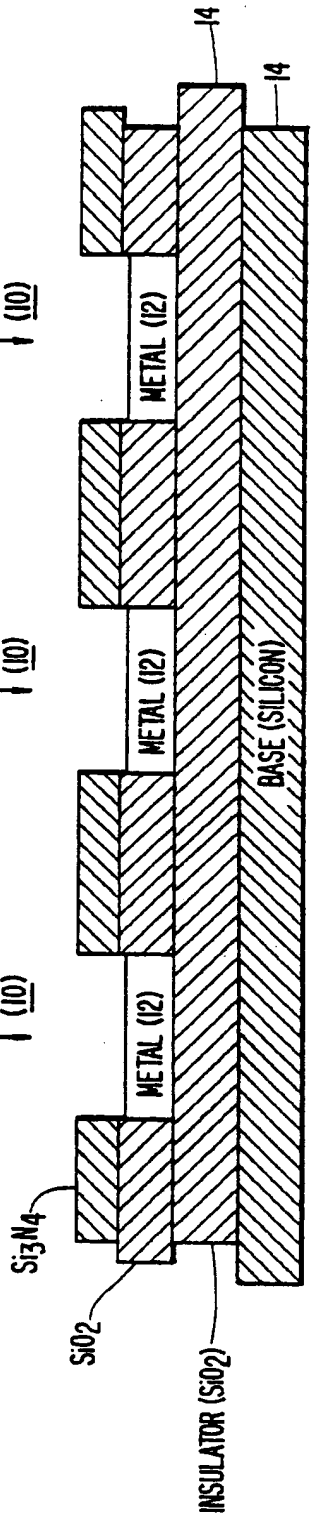

14
14
ML-3
(10)
METAL (12)
ML-2
(10)
METAL (12)
MICROLOCATION 1 (ML-1)
(10)
METAL (12)
BASE (SILICON)
Si_3N_4
SiO_2
INSULATOR (SiO_2)

FIG. 2.

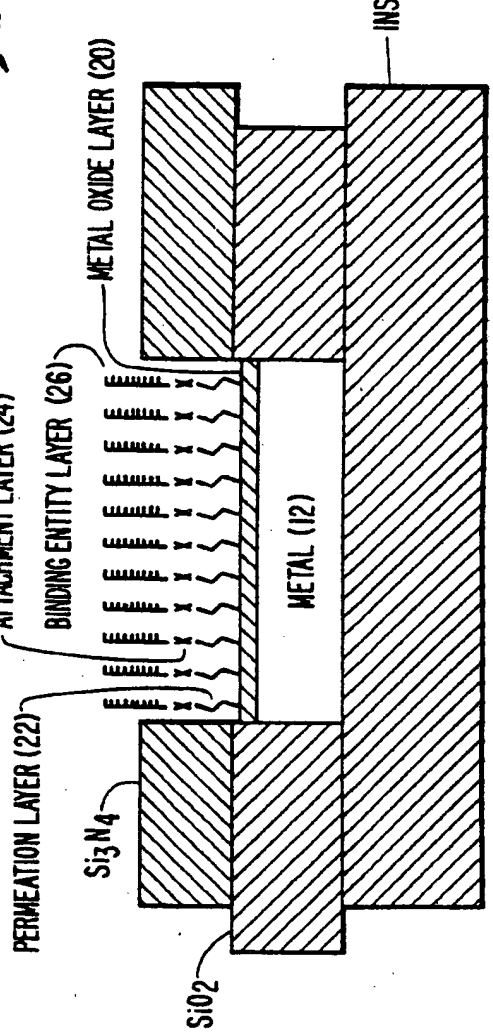
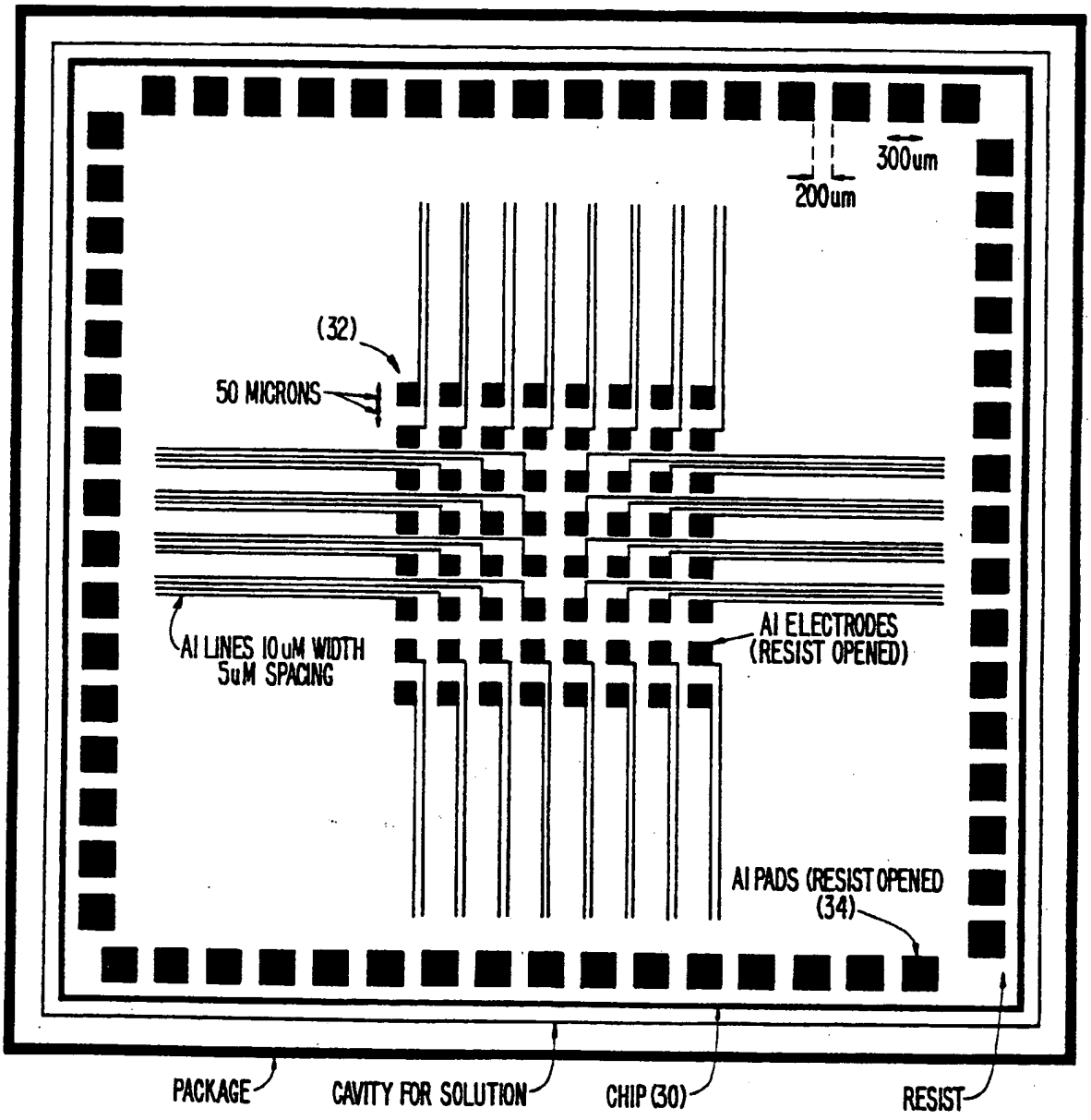

10
INSULATOR (SiO_2)
METAL OXIDE LAYER (20)
ATTACHMENT LAYER (24)
BINDING ENTITY LAYER (26)
METAL (12)
PERMEATION LAYER (22)
Si_3N_4
SiO_2

FIG. 3.


300um
200um
(32)
50 MICRONS
Al LINES 10uM WIDTH
5uM SPACING
Al ELECTRODES
(RESIST OPENED)
Al PADS (RESIST OPENED
(34)
PACKAGE
CAVITY FOR SOLUTION
CHIP (30)
RESIST

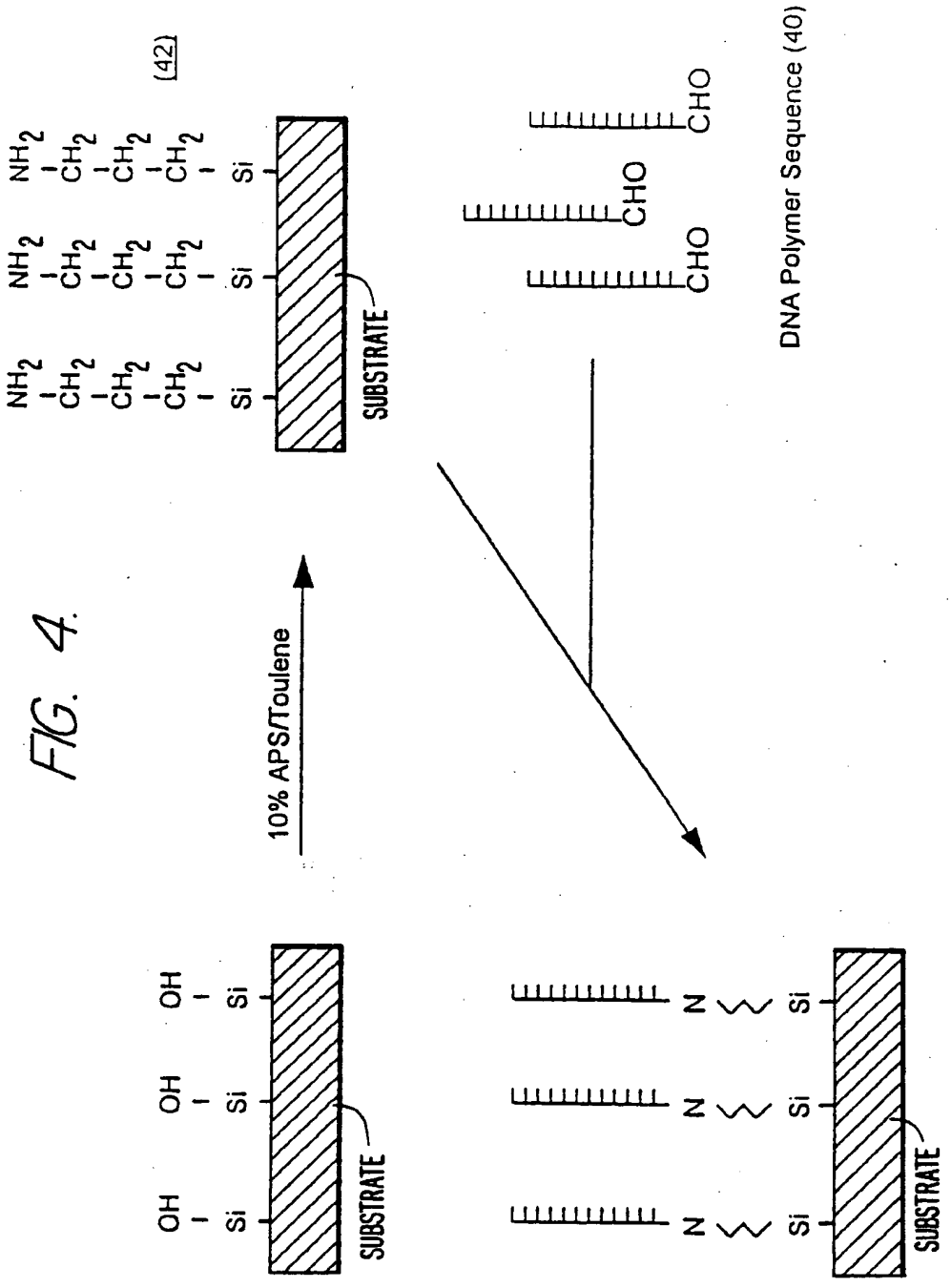

(42)
NH2
CH2
Si
SUBSTRATE
10% APS/Toulene
CHO
DNA Polymer Sequence (40)
OH
N

FIG. 4.

FIG. 5.

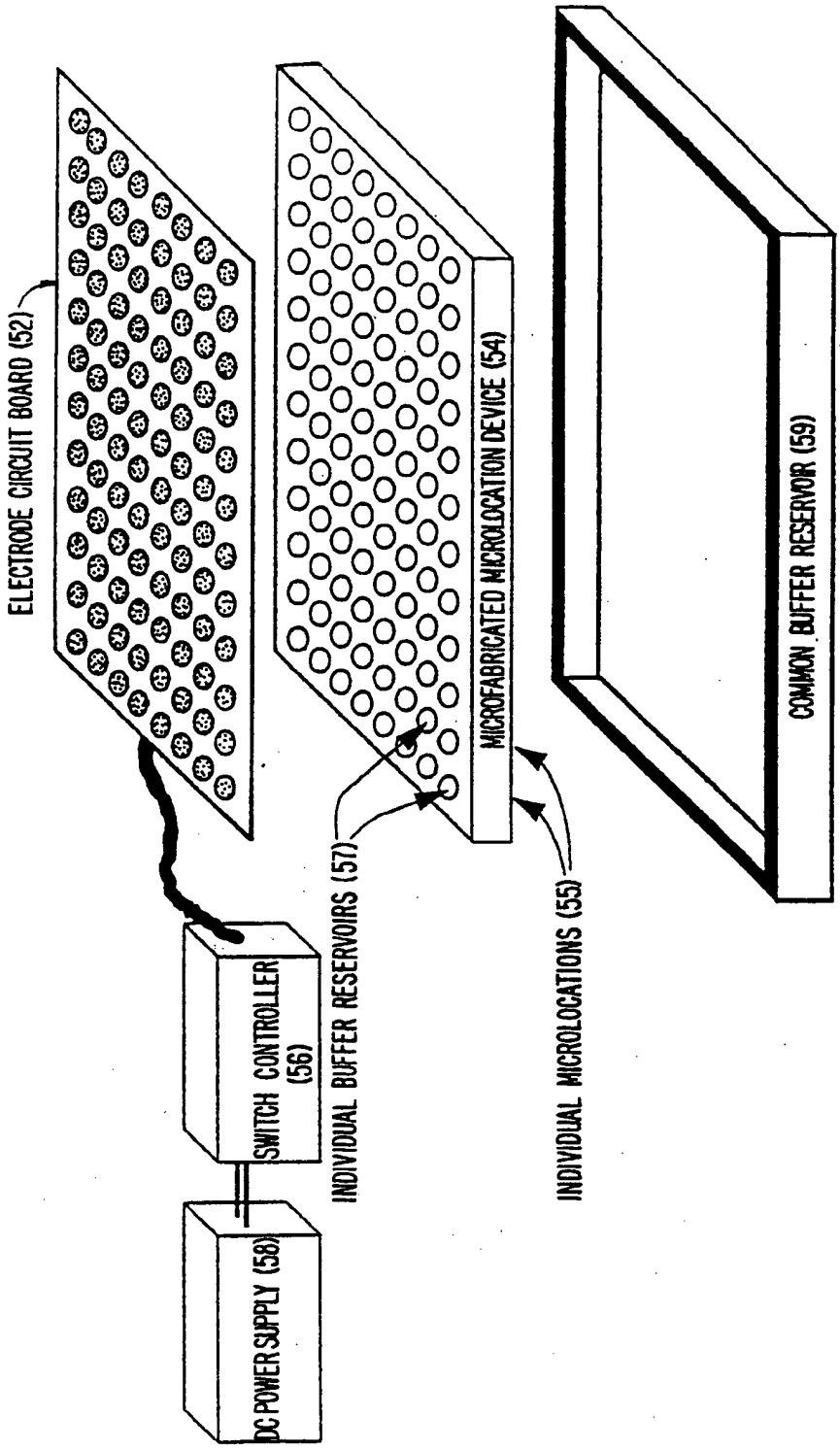
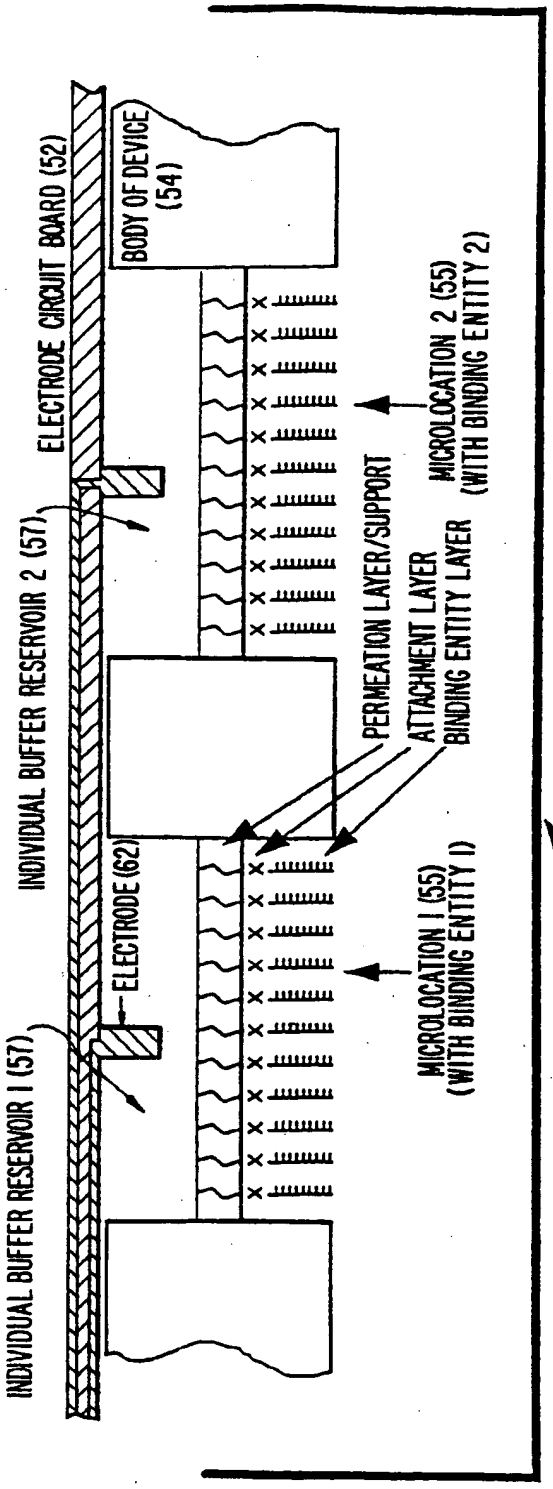

ELECTRODE CIRCUIT BOARD (52)
MICROFABRICATED MICROLOCATION DEVICE (54)
COMMON BUFFER RESERVOIR (59)
INDIVIDUAL BUFFER RESERVOIRS (57)
INDIVIDUAL MICROLOCATIONS (55)
SWITCH CONTROLLER (56)
DC POWER SUPPLY (58)

FIG. 6.


BODY OF DEVICE (54)
ELECTRODE CIRCUIT BOARD (52)
INDIVIDUAL BUFFER RESERVOIR 2 (57)
MICROLOCATION 2 (55) (WITH BINDING ENTITY 2)
PERMEATION LAYER/SUPPORT
ATTACHMENT LAYER
BINDING ENTITY LAYER
ELECTRODE (62)
INDIVIDUAL BUFFER RESERVOIR 1 (57)
MICROLOCATION 1 (55) (WITH BINDING ENTITY 1)
COMMON BUFFER RESERVOIR (59)

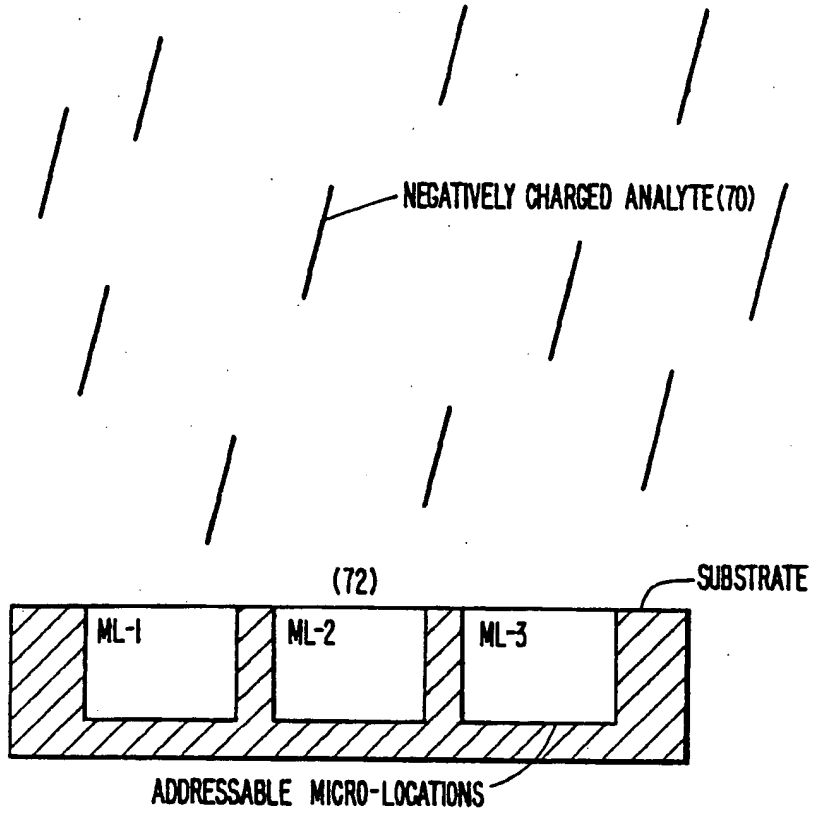

NEGATIVELY CHARGED ANALYTE (70)
(72)
SUBSTRATE
ML-1
ML-2
ML-3
ADDRESSABLE MICRO-LOCATIONS

FIG. 7a.

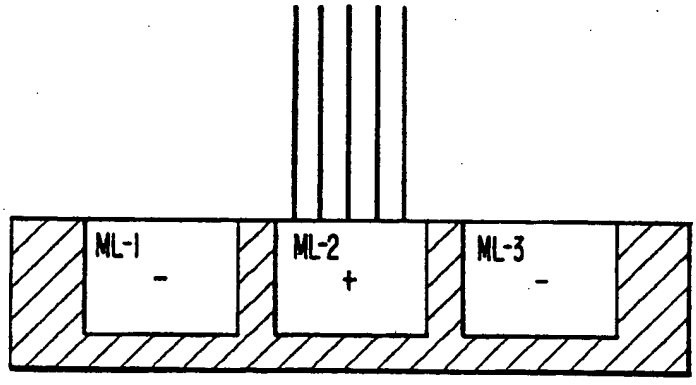

ML-1
-
ML-2
+
ML-3
-

FIG. 7b.

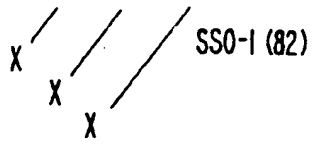


FIG. 8a.

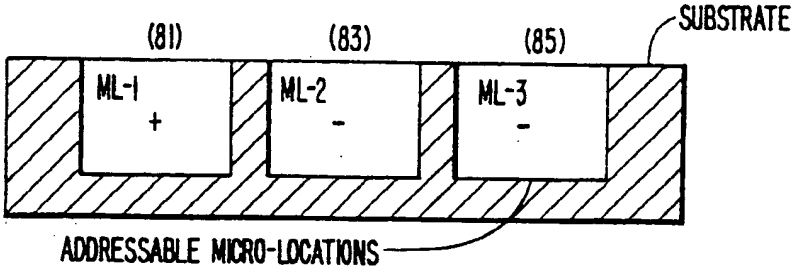
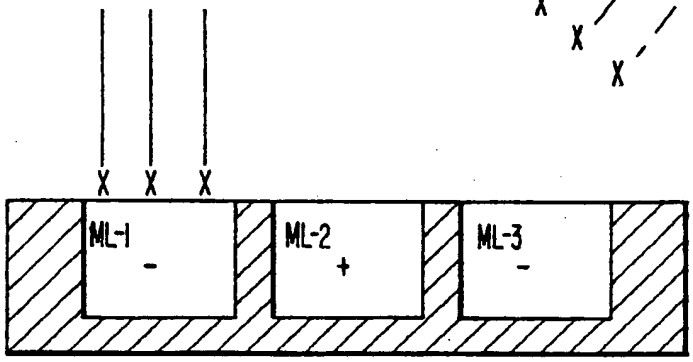


FIG. 8b.



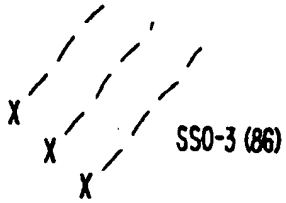


FIG. 8c.

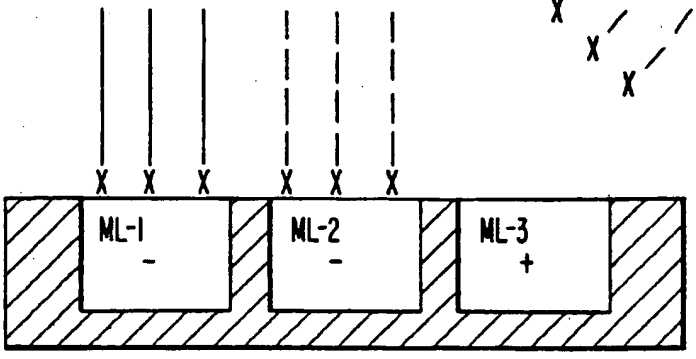


FIG. 8d.

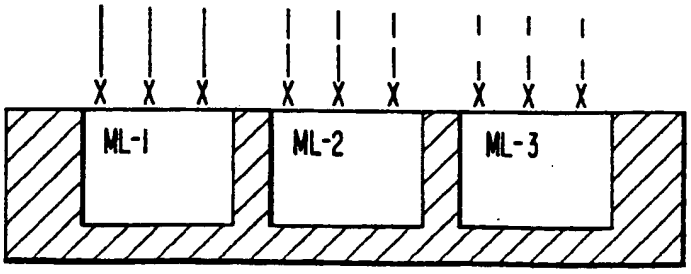


FIG. 9a.

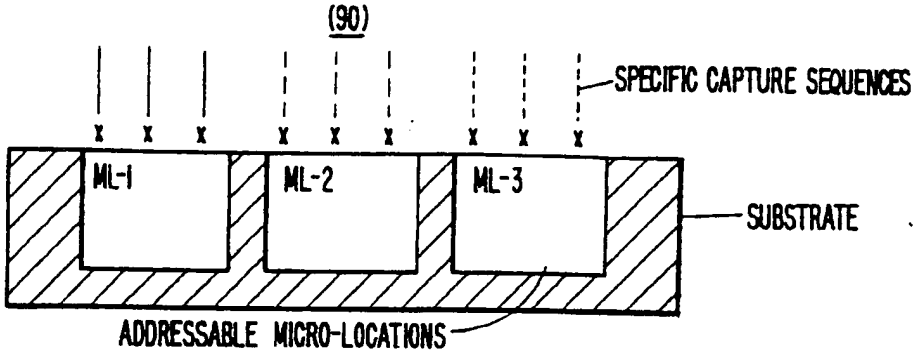


FIG. 9b.

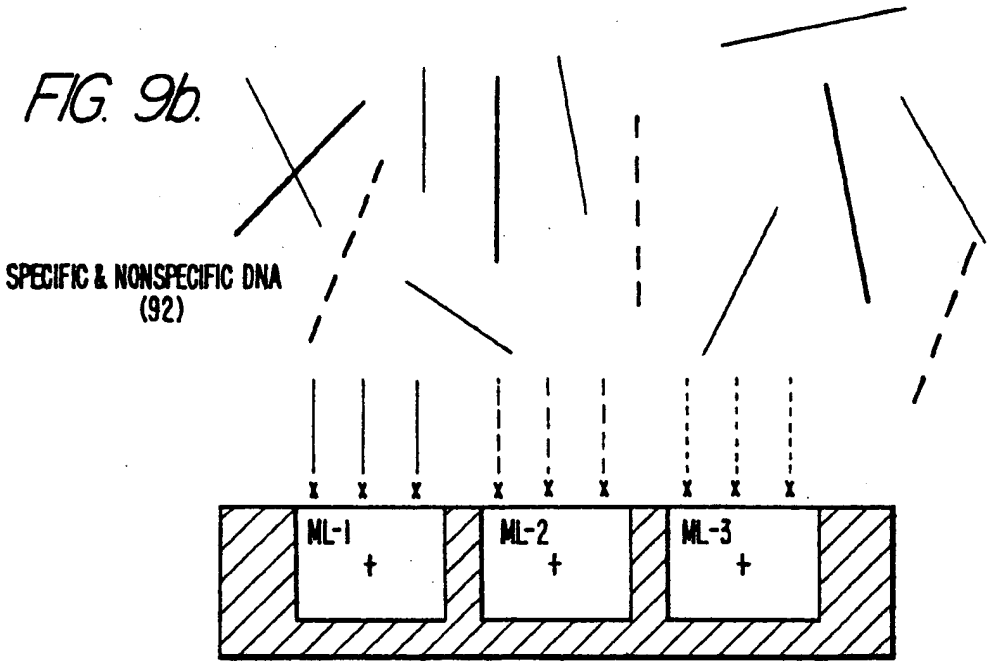
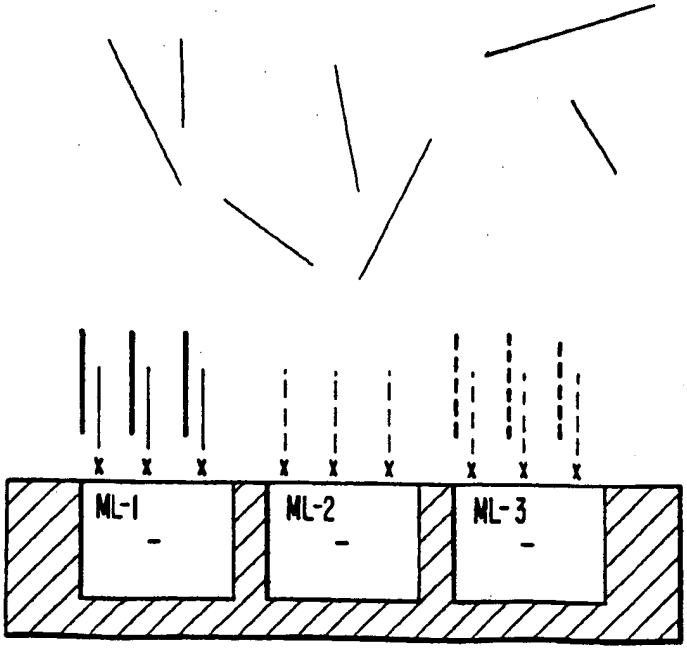


FIG. 9c.



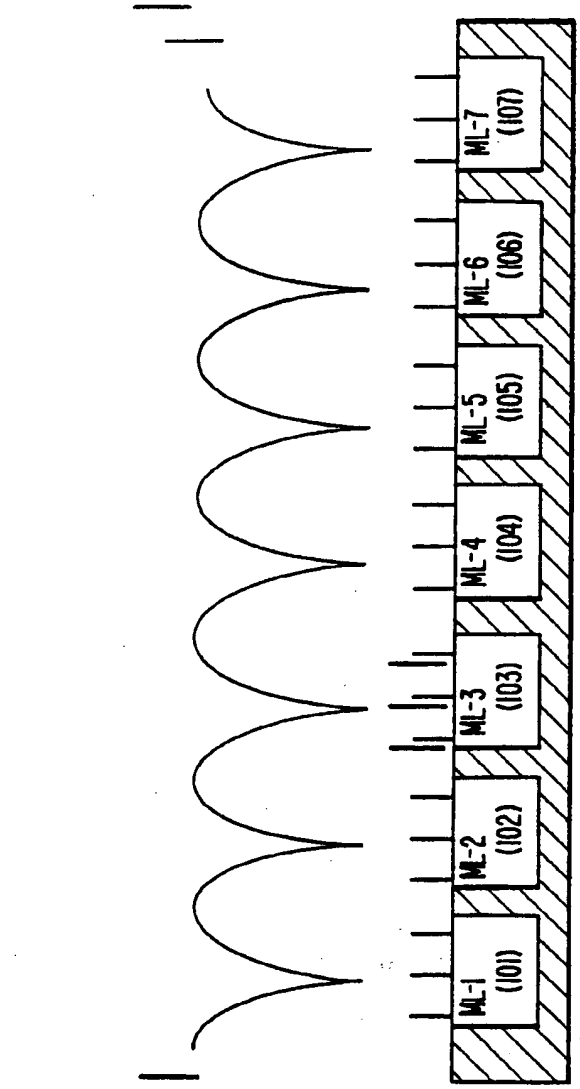


FIG. 10a.

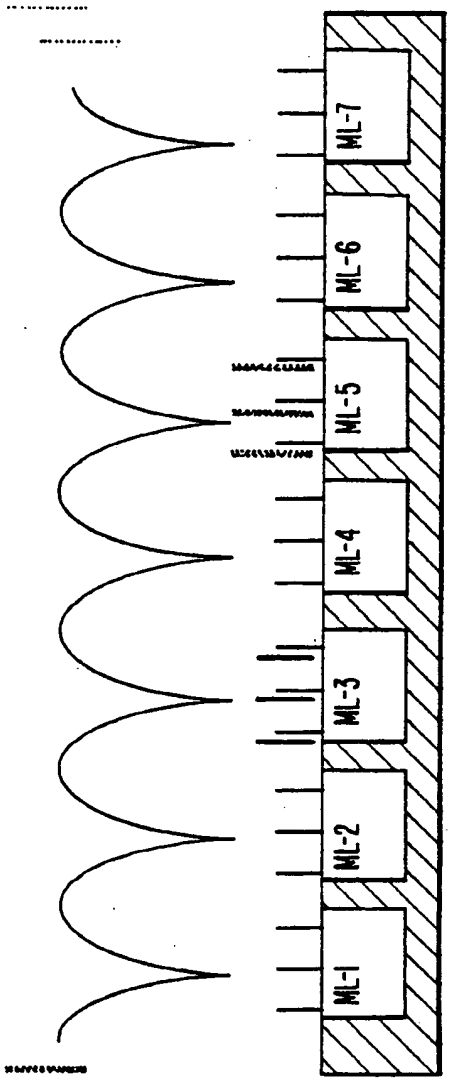


FIG. 10b.

FIG. 11a.

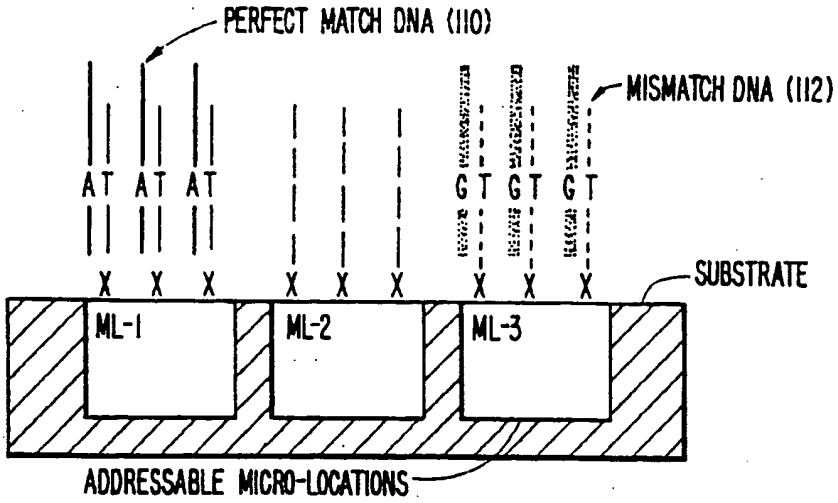


FIG. 11b.

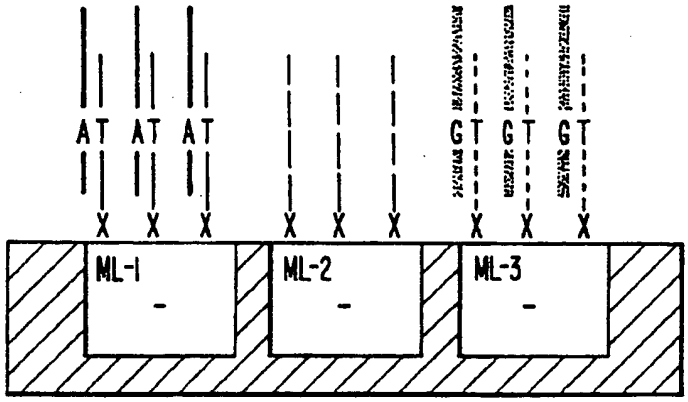


FIG. 11c.

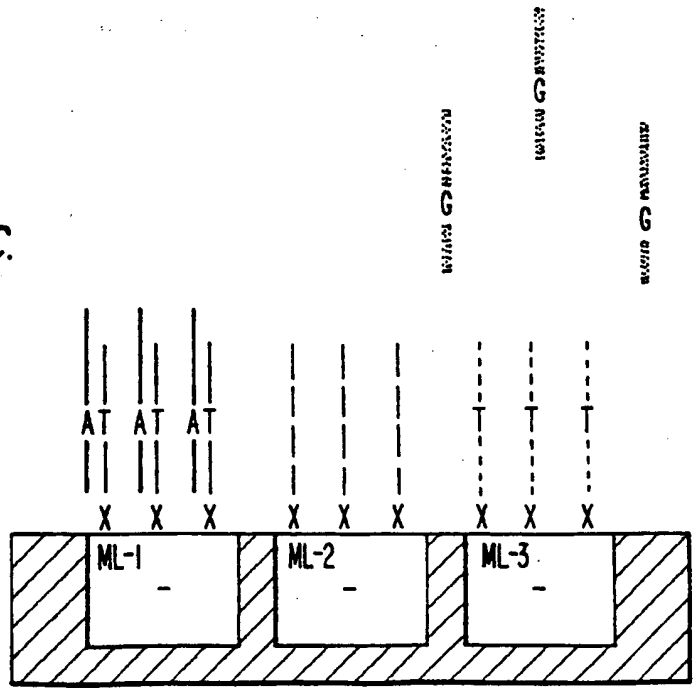


FIG. 12a.

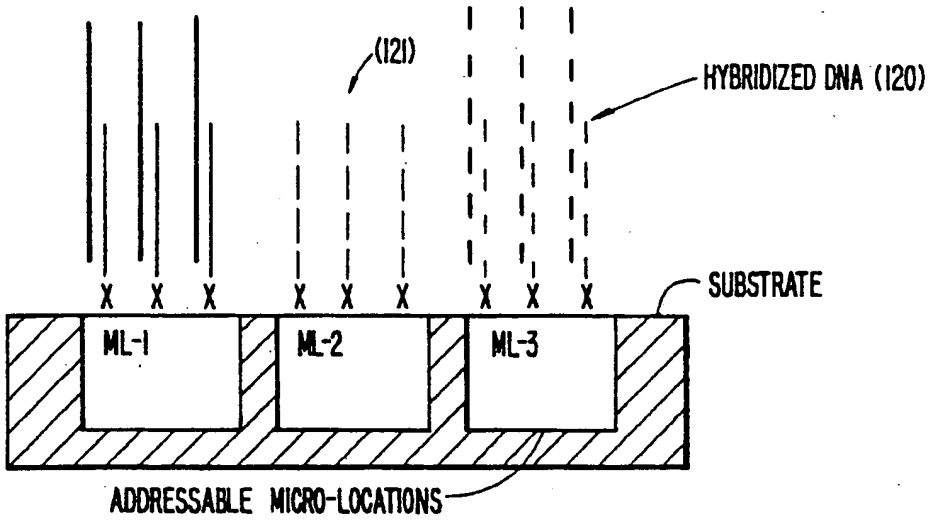


FIG. 12b.

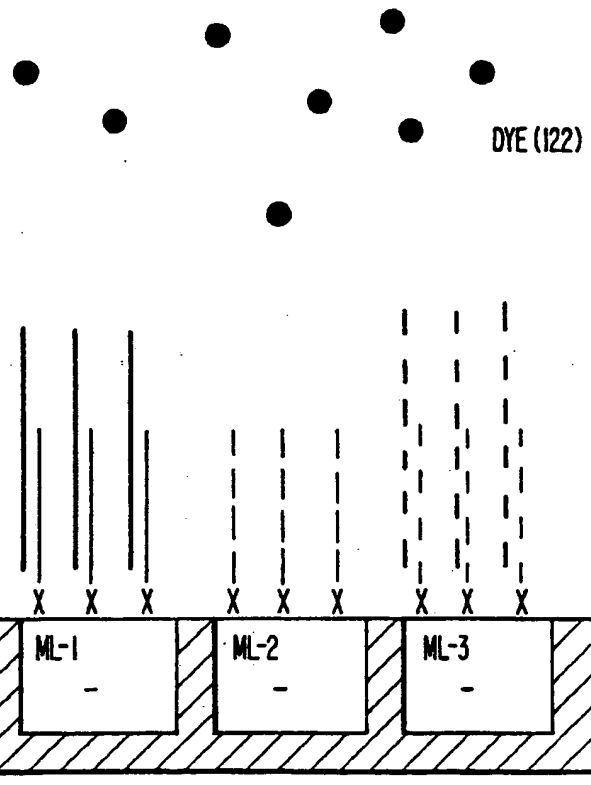


FIG. 12c.

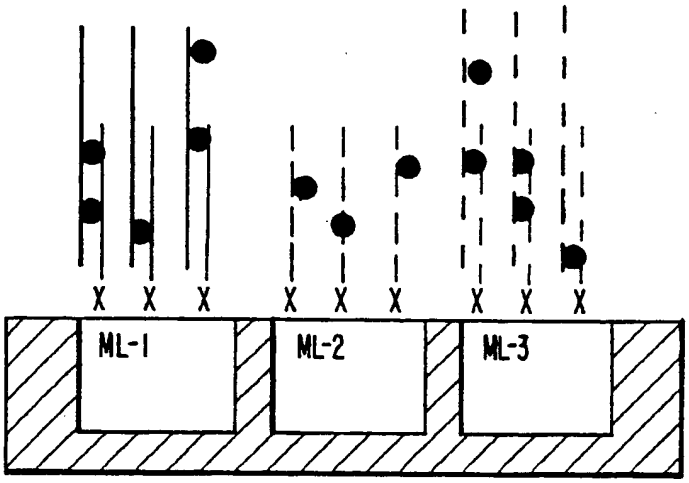


FIG. 12d.

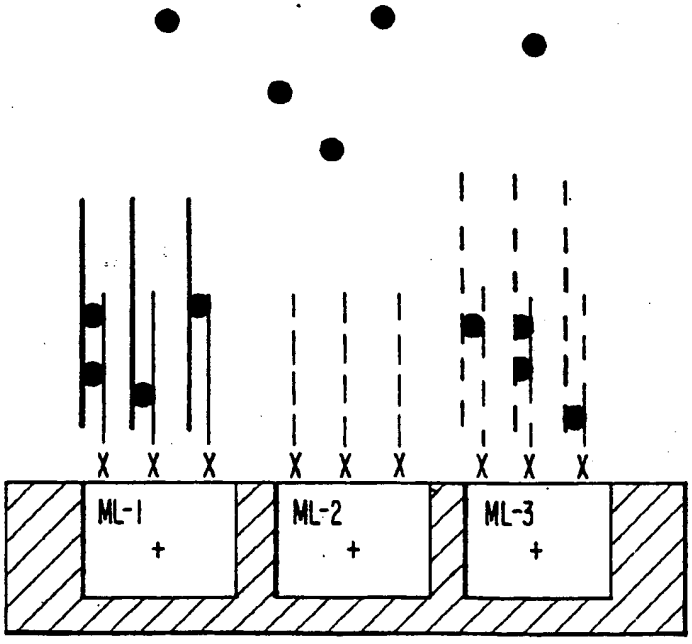


FIG. 13a.

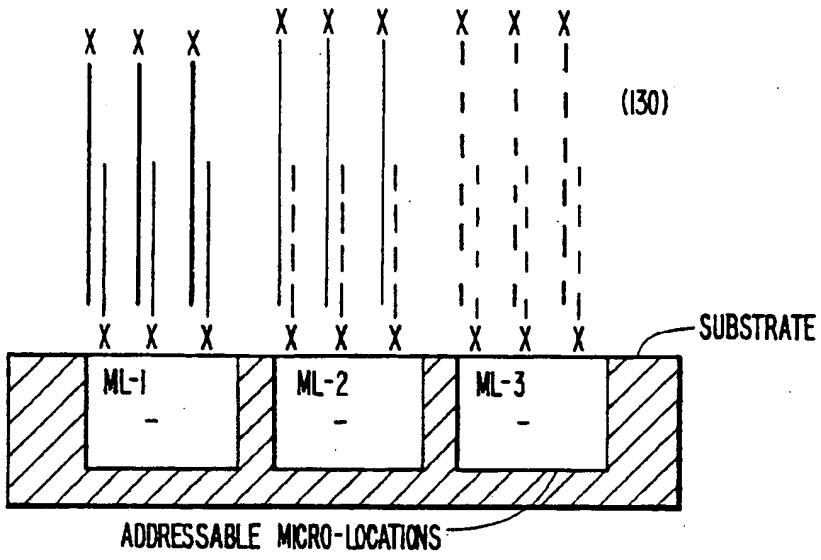


FIG. 13b.

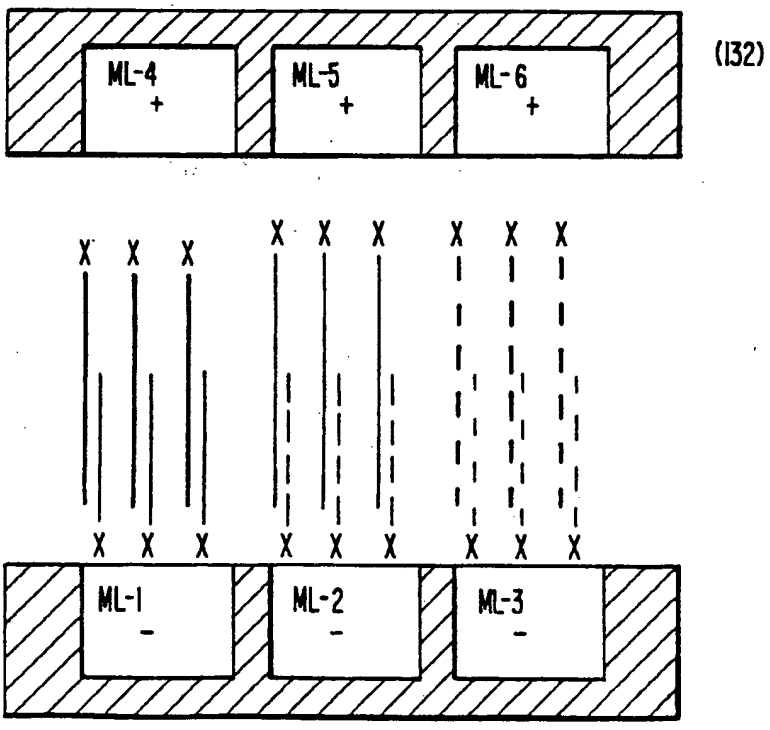


FIG. 13c.

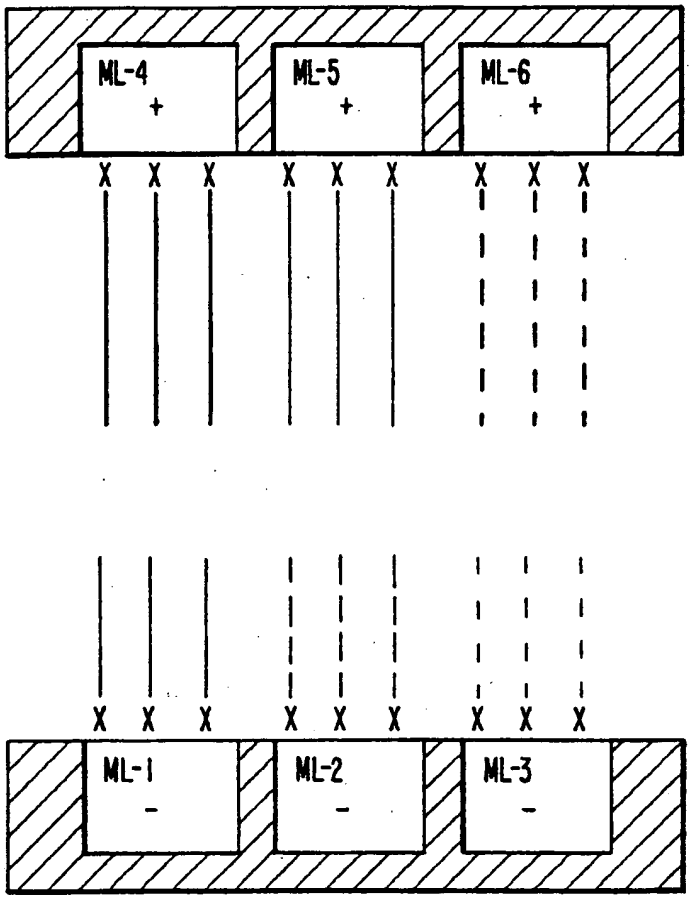


FIG. 14a.

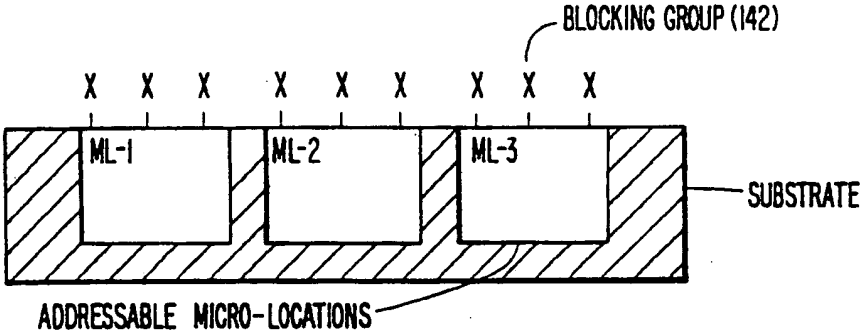


FIG. 14b.

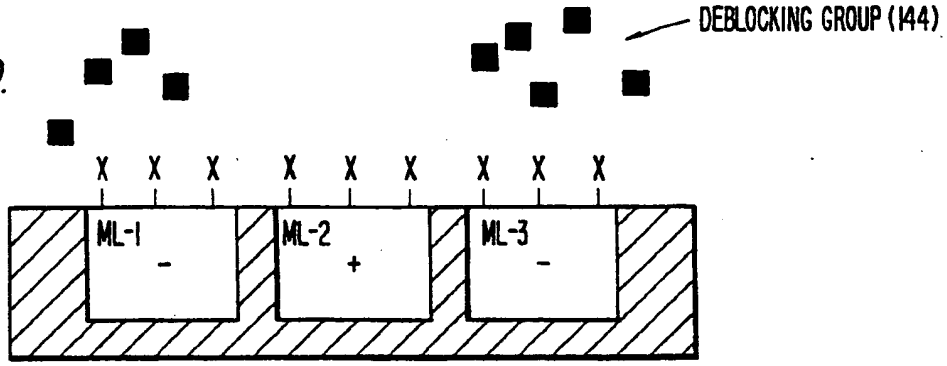


FIG. 14c.

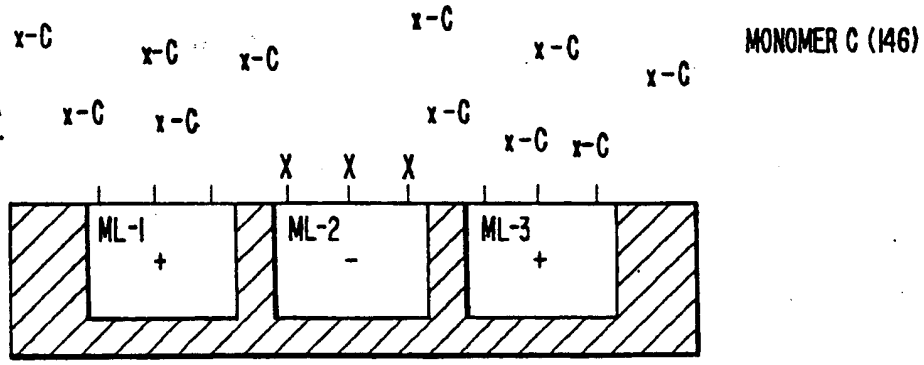


FIG. 14d.

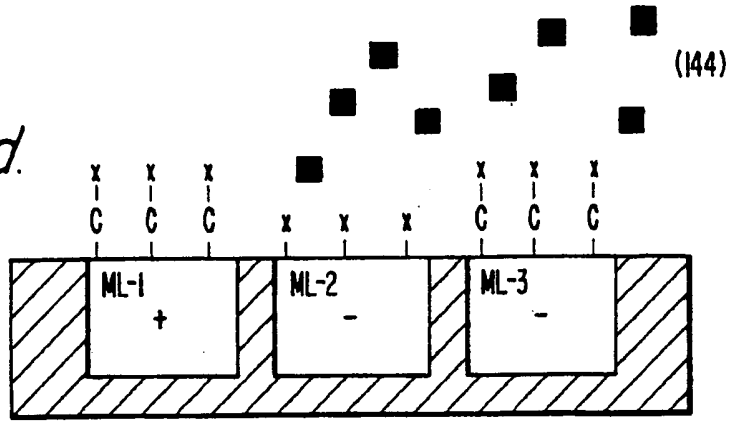


FIG. 14e.

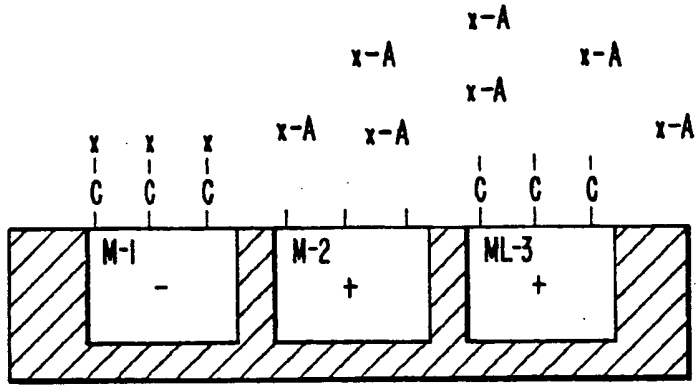
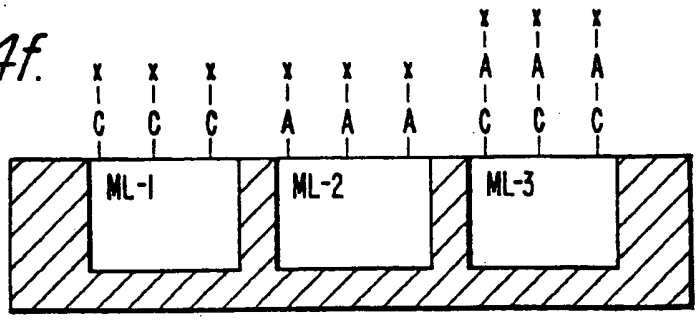


FIG. 14f.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12270

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 21/00, 30/00, 33/53; C07H 21/00; C12Q 1/68
US CL :422/57, 69; 435/6,7.1; 536/25.3, 25.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,227,265 (DEBOER ET AL.) 13 JULY 1993, see especially the abstract and Figures 1-13.	1-11, 13, 16-24, 27-32, 44-48 --- 12, 14, 15, 25, 26, 33, 34
Y	US, A, 3,950,738 (HAYASHI ET AL.) 13 APRIL 1976, see especially the abstract and claims 1-19.	1-34, 44-48
X	US, A, 4,816,418 (MACK ET AL.) 28 MARCH 1989, see especially claims 1-15.	44-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 FEBRUARY 1995	Date of mailing of the international search report 24 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12270

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US, A, 5,075,077 (DURLEY, III ET AL.) 24 DECEMBER 1991, see especially claims 1-24.	1-13, 15-24, 27-32, 44-48 --- 14, 25, 26, 33, 34
X	US, A, 4,580,895 (PATEL) 08 APRIL 1986, see especially claims 1-13.	44-48
Y	US, A, 5,125,748 (BJORNSON ET AL.) 30 JUNE 1992, see especially claims 1-19.	44-48
Y	US, A, 5,164,319 (HAFEMAN ET AL.) 17 NOVEMBER 1992, see especially Figures 1-4C and claims 1-10.	1-34
X — Y	US, A, 5,234,566 (OSMAN ET AL.) 10 AUGUST 1993, see especially the abstract, Figure 2, and claims 1-38.	1-13, 15-24, 27-32 --- 14, 25, 26, 33, 34
X — Y	US, A, 5,096,807 (LEABACK) 17 MARCH 1992, see especially the abstract, Figures 1-7, and claims 1-48.	1-13, 15-24, 27-32 --- 14, 25, 26, 33, 34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12270

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12270

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

422/50, 52, 56, 57, 58, 62, 68.1, 69, 82.01, 82.05, 82.06, 82.07, 82.08, 82.09; 435/4, 5, 6, 7.1, 810; 436/501, 63, 72; 536/25.3, 25.4

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

CAS, BIOSIS, MEDLINE, WORLD PATENT INDEX, BIOTECH ABSTRACTS.
search terms: hybridization, biochip, array, charge, detection, DNA, nucleic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.
Group I, claims 1-34, drawn to electronic devices with a substrate and a permeation layer.

Group II, claims 35-39, drawn to methods of electronically controlling hybridization of DNA.

Group III, claim 40, drawn to a method of actively transporting DNA.

Group IV, claim 41, drawn to an electronically controlled method for combinational synthesis of a biopolymer.

Group V, claims 42 and 43, drawn to a method for replicating a self-addressable electronic device.

Group VI, claims 44-48, drawn to systems for the detection of fluorescent or colorimetric binding reactions and assays.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is directed to devices with a substrate and permeation layer but contain no recited limitation that directs either their making or use to any other group per se. It is noted that binding entities are cited in claim 23, for example, but without any limitation that limits their use or preparation or directs same to another invention group as claimed. Binding entities are also well known and not deemed a special technical feature. Group I therefore lacks a special technical feature that links the claimed devices to any other invention group. Group II is directed to hybridization control but cites no limitation directed to any of Groups III-VI. That is, hybridization is not cited as a special technical feature for the transport of Group III etc. Group III is directed to transport of DNA but does cite synthetic limitations as its use etc. as cited in Groups IV etc. therefore also lacking a special technical feature that links Group III to the other Groups. Group IV is directed to biopolymer synthesis via directing monomers to selected locations on a substrate where a synthetic reaction can occur. Groups V and VI lack any biopolymer synthesis limitations thus causing Group IV to lack a common special technical feature with Groups V and VI. Group V is directed to replication of devices by hybridization reactions. No such hybridization reactions are cited as limitations in Group VI. Therefore Group V lacks a special technical feature in common with Group VI thus supporting a lack of unity. In summary, as discussed above all of Groups I-VI are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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(54) Title: AUTOMATED MOLECULAR BIOLOGICAL DIAGNOSTIC SYSTEM

(57) **Abstract**

Self-addressable, self-assembling microelectronic system for performing molecular diagnosis, analysis, multi-step and multiplex reactions in microscopic formats. Actively controlled reactions include nucleic acid hybridization, immunoassays, clinical diagnosis and multi-step combinatorial biopolymer synthesis. Controller interfaces with user via input/output devices preferably including a graphical display. The controller may interface with a power supply and interface, the interface providing selective connection to individual microlocations, polarity reversal, and selective potential or current levels to individual electrodes. A combined system for performing DNA sample preparation, hybridization, detection and data analysis integrates multiple steps. Charged materials are transported preferably by free field electrophoresis. DNA complexity reduction is preferably achieved by binding DNA to a support, cleaving unbound materials such as by restriction enzymes, and transporting the cleaved fragments. Active, programmable matrix devices include a square matrix pattern with fanned out electrical connections and optional electrical connections beneath specific microlocations resulting in a highly automated DNA diagnostic system.

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DESCRIPTION

Automated Molecular Biological Diagnostic System

Field of the Invention

This invention relates to devices and systems for performing multi-step molecular biological type diagnostic analyses in multiplex formats. More particularly, the molecular biological type analyses include various nucleic acid hybridizations reactions and associated biopolymer synthesis. Additionally, antibody/antigen reactions and other clinical diagnostics can be performed.

Related Application Information

This application is a continuation-in-part of application Serial No. 08/271,882, filed July 7, 1994, which is a continuation-in-part of Serial No. 07/146,504, filed November 1, 1993, both entitled "SELF-ADDRESSABLE SELF-ASSEMBLING MICROELECTRIC SYSTEMS AND DEVICES FOR MOLECULAR BIOLOGICAL ANALYSIS AND DIAGNOSTICS."

Background of the Invention

Molecular biology comprises a wide variety of techniques for the analysis of nucleic acid and protein. Many of these techniques and procedures form the basis of clinical diagnostic assays and tests. These techniques include nucleic acid hybridization analysis, restriction enzyme analysis, genetic sequence analysis, and the separation and purification of nucleic acids and proteins (See, e.g., J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

Most of these techniques involve carrying out numerous operations (e.g., pipetting, centrifugations, electrophoresis)on a large number of samples. They are often complex and time consuming, and generally require a high degree of accuracy. Many a technique is limited in its

application by a lack of sensitivity, specificity, or reproducibility. For example, these problems have limited many diagnostic applications of nucleic acid hybridization analysis.

The complete process for carrying out a DNA hybridization analysis for a genetic or infectious disease is very involved. Broadly speaking, the complete process may be divided into a number of steps and substeps (see Figure 1). In the case of genetic disease diagnosis, the first step involves obtaining the sample (blood or tissue). Depending on the type of sample, various pre-treatments would be carried out. The second step involves disrupting or lysing the cells, which then release the crude DNA material along with other cellular constituents. Generally, several sub-steps are necessary to remove cell debris and to purify further the crude DNA. At this point several options exist for further processing and analysis. One option involves denaturing the purified sample DNA and carrying out a direct hybridization analysis in one of many formats (dot blot, microbead, microtiter plate, etc.). A second option, called Southern blot hybridization, involves cleaving the DNA with restriction enzymes, separating the DNA fragments on an electrophoretic gel, blotting to a membrane filter, and then hybridizing the blot with specific DNA probe sequences. This procedure effectively reduces the complexity of the genomic DNA sample, and thereby helps to improve the hybridization specificity and sensitivity. Unfortunately, this procedure is long and arduous. A third option is to carry out the polymerase chain reaction (PCR)or other amplification procedure. The PCR procedure amplifies (increases) the number of target DNA sequences. Amplification of target DNA helps to overcome problems related to complexity and sensitivity in genomic DNA analysis. All these procedures are time consuming, relatively complicated, and add significantly to the cost of a diagnostic test. After these sample preparation and DNA processing steps, the

actual hybridization reaction is performed. Finally, detection and data analysis convert the hybridization event into an analytical result.

The steps of sample preparation and processing have typically been performed separate and apart from the other main steps of hybridization and detection and analysis. Indeed, the various substeps comprising sample preparation and DNA processing have often been performed as a discrete operation separate and apart from the other substeps. Considering these substeps in more detail, samples have been obtained through any number of means, such as obtaining of full blood, tissue, or other biological fluid samples. In the case of blood, the sample is processed to remove red blood cells and retain the desired nucleated (white) cells. This process is usually carried out by density gradient centrifugation. Cell disruption or lysis is then carried out, preferably by the technique of sonication, freeze/thawing, or by addition of lysing reagents. Crude DNA is then separated from the cellular debris by a centrifugation step. Prior to hybridization, double-stranded DNA is denatured into single-stranded form. Denaturation of the double-stranded DNA has generally been performed by the techniques involving heating (>Tm), changing salt concentration, addition of base (NaOH), or denaturing reagents (urea, formamide, etc.). Workers have suggested denaturing DNA into its single-stranded form in an electrochemical cell. The theory is stated to be that there is electron transfer to the DNA at the interface of an electrode, which effectively weakens the double-stranded structure and results in separation of the strands. See, generally, Stanley, "DNA Denaturation by an Electric Potential", U.K. patent application 2,247,889 published March 18, 1992.

Nucleic acid hybridization analysis generally involves the detection of a very small number of specific target nucleic acids (DNA or RNA) with an excess of probe DNA, among a relatively large amount of complex non-target

nucleic acids. The substeps of DNA complexity reduction in sample preparation have been utilized to help detect low copy numbers (i.e. 10,000 to 100,000) of nucleic acid targets. DNA complexity is overcome to some degree by amplification of target nucleic acid sequences using polymerase chain reaction (PCR). (See, M.A. Innis et al, PCR Protocols: A Guide to Methods and Applications, Academic Press, 1990). While amplification results in an enormous number of target nucleic acid sequences that improves the subsequent direct probe hybridization step, amplification involves lengthy and cumbersome procedures that typically must be performed on a stand alone basis relative to the other substeps. Substantially complicated and relatively large equipment is required to perform the amplification step.

The actual hybridization reaction represents the most important and central step in the whole process. The hybridization step involves placing the prepared DNA sample in contact with a specific reporter probe, at a set of optimal conditions for hybridization to occur to the target DNA sequence. Hybridization may be performed in any one of a number of formats. For example, multiple sample nucleic acid hybridization analysis has been conducted on a variety of filter and solid support formats (See G. A. Beltz et al., in Methods in Enzymology, Vol. 100, Part B, R. Wu, L. Grossman, K. Moldave, Eds., Academic Press, New York, Chapter 19, pp. 266-308, 1985). One format, the so-called "dot blot" hybridization, involves the non-covalent attachment of target DNAs to filter, which are subsequently hybridized with a radioisotope labelled probe(s). "Dot blot" hybridization gained widespread use, and many versions were developed (see M. L. M. Anderson and B. D. Young, in Nucleic Acid Hybridization - A Practical Approach, B. D. Hames and S. J. Higgins, Eds., IRL Press, Washington, D.C. Chapter 4, pp. 73-111, 1985). It has been developed for multiple analysis of genomic mutations (D. Nanibhushan and D. Rabin, in EPA 0228075,

July 8, 1987) and for the detection of overlapping clones and the construction of genomic maps (G. A. Evans, in US Patent Number 5,219,726, June 15, 1993).

New techniques are being developed for carrying out multiple sample nucleic acid hybridization analysis on micro-formatted multiplex or matrix devices (e.g., DNA chips) (see M. Barinaga, 253 Science, pp. 1489, 1991; W. Bains, 10 Bio/Technology, pp. 757-758, 1992). These methods usually attach specific DNA sequences to very small specific areas of a solid support, such as micro-wells of a DNA chip. These hybridization formats are micro-scale versions of the conventional "dot blot" and "sandwich" hybridization systems.

The micro-formatted hybridization can be used to carry out "sequencing by hybridization" (SBH) (see M. Barinaga, 253 Science, pp. 1489, 1991; W. Bains, 10 Bio/Technology, pp. 757-758, 1992). SBH makes use of all possible n-nucleotide oligomers (n-mers) to identify n-mers in an unknown DNA sample, which are subsequently aligned by algorithm analysis to produce the DNA sequence (R. Drmanac and R. Crkvenjakov, Yugoslav Patent Application #570/87, 1987; R. Drmanac et al., 4 Genomics, 114, 1989; Strezoska et al., 88 Proc. Natl. Acad. Sci. USA 10089, 1992; and R. Dramanac and R. B. Crkvenjakov, U.S. Patent #5,202,231, April 13, 1993).

There are two formats for carrying out SBH. The first format involves creating an array of all possible n-mers on a support, which is then hybridized with the target sequence. The second format involves attaching the target sequence to a support, which is sequentially probed with all possible n-mers. Both formats have the fundamental problems of direct probe hybridizations and additional difficulties related to multiplex hybridizations.

Southern, United Kingdom Patent Application GB 8810400, 1988; E. M. Southern et al., 13 Genomics 1008, 1992, proposed using the first format to analyze or sequence DNA. Southern identified a known single point

mutation using PCR amplified genomic DNA. Southern also described a method for synthesizing an array of oligonucleotides on a solid support for SBH. However, Southern did not address how to achieve optimal stringency condition for each oligonucleotide on an array.

Concurrently, Drmanac et al., 260 Science 1649-1652, 1993, used the second format to sequence several short (116 bp) DNA sequences. Target DNAs were attached to membrane supports ("dot blot" format). Each filter was sequentially hybridized with 272 labelled 10-mer and 11-mer oligonucleotides. A wide range of stringency condition was used to achieve specific hybridization for each n-mer probe; washing times varied from 5 minutes to overnight, and temperatures from 0°C to 16°C. Most probes required 3 hours of washing at 16°C. The filters had to be exposed for 2 to 18 hours in order to detect hybridization signals. The overall false positive hybridization rate was 5% in spite of the simple target sequences, the reduced set of oligomer probes, and the use of the most stringent conditions available.

A variety of methods exist for detection and analysis of the hybridization events. Depending on the reporter group (fluorophore, enzyme, radioisotope, etc.) used to label the DNA probe, detection and analysis are carried out fluorometrically, colorimetrically, or by autoradiography. By observing and measuring emitted radiation, such as fluorescent radiation or particle emission, information may be obtained about the hybridization events. Even when detection methods have very high intrinsic sensitivity, detection of hybridization events is difficult because of the background presence of non-specifically bound materials. A number of other factors also reduce the sensitivity and selectivity of DNA hybridization assays.

Attempts have been made to combine certain processing steps or substeps together. For example, various microrobotic systems have been proposed for preparing arrays of DNA probe on a support material. For example,

Beattie et al., in The 1992 San Diego Conference: Genetic Recognition, November, 1992, used a microrobotic system to deposit micro-droplets containing specific DNA sequences into individual microfabricated sample wells on a glass substrate.

Generally, the prior art processes have been extremely labor and time intensive. For example, the PCR amplification process is time consuming and adds cost to the diagnostic assay. Multiple steps requiring human intervention either during the process or between processes is suboptimal in that there is a possibility of contamination and operator error. Further, the use of multiple machines or complicated robotic systems for performing the individual processes is often prohibitive except for the largest laboratories, both in terms of the expense and physical space requirements.

As is apparent from the preceding discussion, numerous attempts have been made to provide effective techniques to conduct multi-step, multiplex molecular biological reactions. However, for the reasons stated above, these techniques are "piece-meal" and limited. These various approaches are not easily combined to form a system which can carry out a complete DNA diagnostic assay. Despite the long-recognized need for such a system, no satisfactory solution has been proposed previously.

Summary of the Invention

The present invention relates to the design, fabrication, and uses of a self-addressable self-assembling microelectronic devices and systems which can actively carry out controlled multi-step processing and multiplex reactions in a microscopic formats. These reactions include, but are not limited to, most molecular biological procedures, such as nucleic acid hybridization, antibody/antigen reaction, and related clinical diagnostics. In addition, the claimed devices and systems are able to carry out multi-step combinational biopolymer synthesis,

including, but not limited to, the synthesis of different oligonucleotides or peptides at specific micro-locations on a given device.

The claimed devices and systems are fabricated using both microlithographic and micro-machining techniques. The basic device has a matrix of addressable microscopic locations on its surface; each individual micro-location is able to control electronically and direct the transport and attachment of specific binding entities (e.g., nucleic acids, enzymes, antibodies) to itself. All micro-locations can be addressed with their specific binding entities. The self-addressing process requires minimal outside intervention in terms of fluidics or mechanical components.

The device is able to control and actively carry out a variety of assays and reactions. Analytes or reactants can be transported by free field electrophoresis to any specific micro-location where the analytes or reactants are effectively concentrated and reacted with the specific binding entity at the micro-location. In the case of hybridization analysis, the sensitivity for detecting a specific analyte or reactant is improved because hybridization reactants are concentrated at a specific microscopic location. Any un-bound analyltes or reactants can be removed by reversing the polarity of a micro-location. Thus, the device also improves the specificity of the reactions. Basic devices for nucleic acid hybridization and other analyses are alternatively referred to as APEX devices, which stands for addressable programmable electronic matrix.

In one aspect of the invention, additional processing steps or substeps may be performed in sequence with a "system". The system is an integrated arrangement of component devices. Each component device is appropriately designed and scaled to carry out a particular function. In its most complete embodiment, a system may perform all aspects of sample preparation, hybridization and detection

and analysis. In this fullest form, the sample is first prepared, such as by an electronic cell sorter component. Generally, electronic refers more specifically to the ability of the component device to electrophoretically transport charged entities to or from itself. Further DNA processing and complexity reduction may optionally be performed by a crude DNA selector component, and a restriction fragment selector component. The final processed target DNA is transported to the analytical component where electronic hybridization analysis is carried out in a microscopic multiplex format. This analytical component device is also referred to as the APEX or analytical chip. Associated detection and image analysis components provide the results.

Within the system materials may optionally be transported between components (devices) by free field electrophoresis, channelling, fluidics or other techniques. Optionally, electronic reagent dispenser components can provide electrophoretic transport of reagents to the various processing components of the system. Optionally, an electronic waste disposal system may be formed by providing an electrode and charged matrix material that attracts and holds charged waste products. Optionally, an electronic DNA fragment storage system can serve to temporarialy hold other DNA fragments for later hybridization analysis.

In one aspect of this invention, genomic DNA complexity reduction is performed by processes that isolate those specific DNA fragments containing the desired target sequence from the bulk of the DNA material that lacks the desired target sequence. Crude DNA can be transported and captured on a support material. The bound DNA can then be severed using appropriate restriction enzymes. After severing, the DNA fragments can be transported to a component device that selectively hybridizes specific DNA fragments. Those fragments that contain the actual target sequences to be analyzed can be selectively released, via further restriction enzyme cleavage, and transported to

the analytical component (APEX chip) of the system. Optionally, this procedure may be repeated for other fragments containing other target sequences.

A controller for the device (or system) provides for individual control of various aspects of the device. When an APEX device or chip containing addressable microscopic locations is utilized, the controller permits individual microlocations to be controlled electronically so as to direct the transport and attachment of specific binding entities to that location. The device may carry out multi-step and multiplex reactions with complete and precise electronic control, preferably under control of a microprocessor based component. The rate, specificity, and sensitivity of multi-step and multiplex reactions are greatly improved at the specific microlocations on the device. The controller interfaces with a user via input/output devices, such as a display and keyboard input. Preferably, a graphical user interface is adapted for ease of use. The input/output devices are connected to a controller, which in turn controls the electrical status of the addressable electronic locations on the system. Specifically, the controller directs a power supply/waveform generator to generate the electronic status of the various microlocations. Optionally, an interface is used between the power supply/waveform generator and the APEX device or system. The interface preferably comprises a bank of relays subject to the controller via a multifunction input/output connection. The relays preferably serve to connect the power supply/waveform generator to the APEX device by controlling the connection as to its polarity, the presence or absence of a connection and the amount of potential or current supply to the individual location. The controller preferably controls the illumination source directed at the hybridization system. A detector, image processing and data analysis system are optically coupled to the APEX device. In the preferred embodiment, a fluorescent microscope receives

and magnifies the image from the hybridization events occurring on the various micro-locations of the device. The emissions are optically filtered and detected by a charge coupled device (CCD) array or microchannel plate detector. The image is then stored and analyzed. Preferably, the results are displayed to the user on the monitor.

In another aspect of this invention, the hybridization system is formed having a plurality of microlocations formed atop a substrate containing control electronics. Specifically, switching circuits are provided to address individually the microlocations. The electrical connections are made via the backside relative to where sample contact is to be made. Additionally, an optical pathway, such as a waveguide, is disposed beneath the microlocation to permit backside access to the microlocation. Optical excitation, if necessary, may be directed to the microlocation via the waveguide. Detection of emitted radiation may be detected via the backside waveguide. In yet another aspect of this invention, a sample containment system is disposed over the system, particularly the hybridization matrix region. In the preferred embodiment, the matrix hybridization region (including sample containment component) is adapted for removal from the remainder of the device providing the electronic control and detector elements.

In another aspect of this invention, improved processes for forming a matrix hybridization system are described. In one process, a substrate, such as silicon, is formed with an insulating layer, such as a thick oxide. Conductive microlocations are formed, such as by deposition of metal (e.g., aluminum or gold) that is then patterned, such as by conventional photolithographic techniques. An insulating coating is formed, such as TEOS formed by PECVD. Optionally, a nitride passivation coating is formed over the TEOS layer. Openings to the microelectrode are formed through the nitride and glass.

Optionally, adhesion improving materials such as titanium tungsten may be utilized in connection with the metal layer to promote adhesion to the oxide and/or glass. In yet a further improvement, wells may be formed atop of the electrode by undercutting a nitride layer disposed on an oxide layer supported by the substrate.

Electronic control of the individual microlocations may be done so as to control the voltage or the current. When one aspect is set, the other may be monitored. For example, when voltage is set, the current may be monitored. The voltage and/or current may be applied in a direct current mode, or may vary with time. For example, pulsed currents or DC biases may be advantageously utilized.

Accordingly, it is an object of this invention to provide a system for the sample preparation, processing, hybridization, detection and analysis of biological materials.

It is yet a further object of this invention to provide a system that combines multiple steps or substeps within an integrated system.

It is yet a further object of this invention to provide for an automated DNA diagnostic system.

Brief Description of the Drawings

Fig. 1 shows the sequence of steps and substeps for sample preparation, hybridization and detection and data analysis.

Figs. 2A and 2B show the active, programmable matrix system in cross-section (Fig. 2A) and in perspective view (Fig. 2B).

Fig. 3 shows the active, programmable matrix system structure at the metal mask layer.

Fig. 4 shows detail of the active, programmable matrix system in plan view.

Fig. 5 shows a perspective view of a single microlocation and electrical connection.

Fig. 6 shows a plan view of the system including an electronic cell sorter matrix, DNA selectors and restriction fragment selectors and hybridization matrix.

Fig. 7 shows a block diagram description of the control system.

Fig. 8 is a cross-sectional view of the active, programmable matrix system having associated electronics.

Fig. 9 is a cross-sectional view of an alternative, layered active, programmable matrix system having electrical and optical access to the backside of the microlocations and a biological containment cover.

Fig. 10 shows a perspective view of the APEX system mounted in a mating carrier.

Figs. 11A-G show process steps in device fabrication.

Fig. 12 shows a fabricated device utilizing a polysilicon structure in cross-section.

Fig. 13 shows a fabricated device utilizing adhesion enhancing layers in cross-section.

Fig. 14 shows a device having an enlarged reservoir space above the electrode.

Fig. 15 shows user displays for various voltage and current regimes.

Fig. 16 shows a cross-sectional view of a DNA purification system.

Fig. 17 shows a cross-sectional view of a capillary array manufacturing system and apparatus.

Fig. 18 shows a perspective view of a micro-location of a concentric structure.

Detailed Description of the Invention

Figs. 2A and 2B illustrate a simplified version of the active programmable electronic matrix hybridization system for use with this invention. Generally, a substrate 10 supports a matrix or array of electronically addressable microlocations 12. For ease of explanation,

the various microlocations in Fig. 2A have been labelled 12A, 12B, 12C and 12D. A permeation layer 14 is disposed above the individual electrodes 12. The permeation layer permits transport of relatively small charged entities through it, but precludes large charged entities, such as DNA, from contacting the electrodes 12 directly. The permeation layer 14 avoids the electrochemical degradation which would occur in the DNA by direct contact with the electrodes 12. It further serves to avoid the strong, non-specific adsorption of DNA to electrodes. Attachment regions 16 are disposed upon the permeation layer 14 and provide for specific binding sites for target materials. The attachment regions 16 have been labelled 16A, 16B, 16C and 16D to correspond with the identification of the electrodes 12A-D, respectively.

In operation, reservoir 18 comprises that space above the attachment regions 16 that contains the desired, as well as undesired, materials for detection, analysis or use. Charged entities 20, such as charged DNA are located within the reservoir 18. In one aspect of this invention, the active, programmable, matrix system comprises a method for transporting the charged material 20 to any of the specific microlocations 12. When activated, a microlocation 12 generates the free field electrophoretic transport of any charged functionalized specific binding entity 20 towards the electrode 12. For example, if the electrode 12A were made positive and the electrode 12D negative, electrophoretic lines of force 22 would run between the electrodes 12A and 12D. The lines of electrophoretic force 22 cause transport of charged binding entities 20 that have a net negative charge toward the positive electrode 12A. Charged materials 20 having a net positive charge move under the electrophoretic force toward the negatively charged electrode 12D. When the net negatively charged binding entity 20 that has been functionalized

contacts the attachment layer 16A as a result of its movement under the electrophoretic force, the functionalized specific binding entity 20 becomes covalently attached to the attachment layer 16A.

It is possible to protect the attachment layers which are not subject to reaction, such as 16B and 16C by making their corresponding electrodes 12B and 12C negative. This results in electrophoretic lines of force emanating from the attachment region 16B (only 16B will be discussed for simplicity, the results being similar for 16C). The electrophoretic force lines 24 serve to drive away negatively charged binding entities 20 from the attachment layer 16B and towards the attachment layer 16A. In this way, a "force field" protection is formed around the attachment layers 16 which it is desired to have nonreactive with the charged molecules 20 at that time.

One highly advantageous result of this system is that charged binding materials 20 may be highly concentrated in regions adjacent to signal attachment layers 16. As can be seen in perspective drawing Fig. 2B, if a individual microlocation 26A is positively charged, and the remaining microlocation are negatively charged, the lines of electrophoretic force will cause transport of the net negatively charged binding entities 20 toward the microlocation 26A. The microlocation 26A is intended to depict the combination in Fig. 2A of the attachment layer 16, the permeation layer 14 and the underlying associated electrode 12. In this way, a method for concentrating and reacting analytes or reactants at any specific microlocation on the device may be achieved. After the attachment of the specific binding entities 20 to the attachment layer 16, the underlying microelectrode 12 may continue to function in a direct current (DC) mode. This unique feature allows relatively dilute charged analytes or reactant molecules free in solution to be rapidly transported, concentrated, and reacted in

a serial or parallel manner at any specific micro-location that is maintained at the opposite charge to the analyte or reactant molecules. This ability to concentrate dilute analyte or reactant molecules at selected microlocations 26 greatly accelerates the reaction rates at these microlocations 26.

After the desired reaction is complete, the electrode 12 may have its potential reversed thereby creating an electrophoretic force in the direction opposite to the prior attractive force. In this way, nonspecific analytes or unreacted molecules may be removed from the microlocation 26. Specific analytes or reaction products may be released from any microlocation 26 and transported to other locations for further analysis; or stored at other addressable locations; or removed completely from the system. This removal or deconcentration of materials by reversal of the field enhances the discrimination ability of the system by resulting in removal of nonspecifically bound materials. By controlling the amount of now repulsive electrophoretic force to nonspecifically bound materials on the attachment layer 16, electronic stringency control may be achieved. By raising the electric potential at the electrode 12 so as to create a field sufficient to remove partially hybridized DNA sequences, thereby permitting identification of single mismatched hybridizations, point mutations may be identified.

Operations may be conducted in parallel or in series at the various attachment layers 16. For example, with reference to Fig. 2A, a reaction may occur first at attachment layer 16A utilizing the potentials as shown. The potential at electrode 12A may be reversed, that is, made negative, and the potential at the adjacent electrode 12B may be made positive. In this way, a series reactions occurs. Materials that were not specifically bound to attachment layer 16A would be transported by electrophoretic force to attachment layer 16B. In this

way, the concentration aspect is utilized to provide high concentrations at that specific attachment layer then subject to the positive electrophoretic force. The concentrated materials may next be moved to an adjacent, or other, attachment layer 16. Alternatively, multiple attachment layers 16 may be deprotected in the sense that there is a net electrophoretic force field emanating from the electrode 12 through the attachment layer 16 out into the reservoir 18. By deprotecting multiple attachment layer 16, multiplex reactions are performed. Each individual site 26 may serve in essence as a separate biological "test tube" in that the particular environment addressed by a given attachment layer 16 may differ from those environments surrounding the other attachment layers 16.

Fig. 3 shows a plan view of the metal mask layer for an active programmable electronic matrix system. A plurality of individual electrodes 30 are formed preferably in an array. For example, an 8 x 8 matrix of individual electrodes 30 is formed. Optionally, additional control or dump pads 32 may be provided to aid in generation of desired electrophoretic fields. The electrodes 30 and pad 32 are connected to contact pads 34. 68 contact pads 34 are shown corresponding to the 64 electrodes 30 and 4 pads 32. Leads 36 connect the electrodes 30 and pads 32 individually to the contacts 34. As shown, a fan-out pattern is used to permit connections from the relatively condensed region of the electrodes 30 and pads 32 to the boundaries 36 of the mask.

Fig. 4 shows an exploded detail plan view of the mask of Fig. 3. The resulting metallized system would appear substantially similar to the masked pattern. The electrodes 30 are shown formed as substantially square structures. The lead lines 36 connect the electrode 30 to the contact pad 34 (Fig. 3). The preferred line width of the lead 36 is 1 to 20 microns.

Fig. 5 shows a perspective view of a single electrode 50. The electrode 50 is connected directly to the lead 52. A permeation layer 54 is disposed above the lead 50. An attachment layer 56 is disposed upon the permeation layer 54.

The permeation layer in microlithographically produced devices can range in thickness from 1 nm to 500 micrometers, with 500 nm to 50 micrometers being the most perferred. The permeation layer should cover the entire electrode surface. The permeation layer may be formed from any suitable material such as polymers, ceramics, sol-gels, layered composite materials, clays and controlled porousity glass.

Fig. 6 shows a complete system 60 for the automated sample preparation and hybridization of prepared materials. A sample 62, such as blood or other biological materials are introduced into the system 60. Generally, a sample addition port 64 is provided. Generally, the sample addition port 64 is utilized when an overlying biological containment structure is present such that the sample 62 could not be directly placed into the system without access via the port 64.

Sample preparation is performed in this system 60 by the combination of the electronic cell sorter matrix component 66 and DNA selector component 68 and restriction fragment selector component 70. The electronic cell sorter matrix component 66 consists of underlying electrodes, with permeation layers and an attachment layers. These effectively form a matrix of locations for the attachment of cells. Generally, the area for individual locations and the complete matrix area are larger than the areas in an analytical device component. Thus, the electronic cell sorter matrix is scaled appropriately to accommodate variation in the number of cells from different samples and sample sizes. The attachment layers can be generally selective for cells, or individual selective for different types of cells. Optionally,

groups oe sets of locations can be made selective for one type of cell. Cell selectivity can be imparted by attaching specific antibodies or cell adhesion factors to the attachment layer. The matrix 66 operates by free field electrophoresis.

The crude DNA selector 68 and restriction fragment selector 70 serve to bind the crude DNA output from the electronic cell sorter matrix 66 and permit selective cleavage of the desired DNA from the bound material. The term crude is used merely to denote a non-final stage in DNA isolation or complexity reduction. The DNA is bound to the selector in a region which is believed not to contain the desired DNA material. The desired DNA materials are then severed from the bound materials, such as by application of restriction enzymes. The severed, unbound material is then physically moved from the crude DNA selector 68 to the restriction fragment selector 70. Preferably, electrophoretic transport is used to remove the severed material. This process may be repeated by binding the severed material to a selector, upon which a restriction enzyme acts so as to cleave the unbound portion which contains the desired DNA.

For example, human DNA contains approximately 100,-000 genes. Of the total DNA material, a significant portion constitutes repeating sequences which do not contain the desired DNA information. The DNA may be bound to a selector by these noninformation bearing repeating sequences. The bound DNA may be severed from the unbound DNA which is believed to contain the desired DNA to be analyzed. This process may then be repeated with yet more specific sequences causing binding of the material to the selector.

The output of the restriction fragment selector 70 is then supplied to the APEX chip 72. Operations on the matrix 72 are performed as described in connection with Figs. 2A and 2B.

An alternative technique for reducing DNA complexity is to use DNA-based affinity chromatography. The affinity that a piece of single stranded DNA has for another single stranded piece of DNA depends on how closely the base pairs match. When the stationary phase of a chromatographic system contains a particular sequence or collection of sequences, any single stranded DNA in the mobile phase will adhere to the stationary phase more or less well depending on how closely the sequence matches the capture sequence/s in the stationary phase. This allows chromatographic separations based on the affinity of DNA for capture sequences in the stationary phase.

One method to implement DNA-based affinity chromatography with a matrix of micro-locations is to modify a series of locations with capture probe of a particular sequence or set of sequences. This forms the stationary phase. A sample of DNA is addressed to a microlocation and is moved serially from one micro-location to the next. Electronic stringency control is used to retain the DNA that matches the capture probe well at each micro-location. In this way, DNA that matches the capture probe will be removed rapidly from the sample.

The invention of serial purification of a DNA sample by DNA-based affinity chromatography on a series of micro-locations can be generalized to a continuous version. Fig. 16 shows a cross-sectional view of such a system. Here, the electrode 210 forms a long strip and is modified by an appropriate stationary phase. The mobile phase is confined to a channel 212 above the stationary phase. The mobile phase can be passed over the stationary phase by convective mass transport. Alternatively, ions in the mobile phase can be pulled along the stationary phase by an electric field 214 established by placing separate and independent electrodes 216 at either end of the long strip electrode. Electronic stringency can be used by applying an alter-

nating or pulsed current at the strip electrode. This drives DNA on and off of the stationary phase.

A further alternative method for reducing the complexity of a sample of DNA, is to size select by sieving the sample through a microporous media. Microporous media can be formed by filling cavities of arbitrary geometry with dendrites. These dendrites are formed by electrochemical deposition of chemicals such as, but not exclusive to, metal salts, ceramic forming materials, monomers and polymers. The porosity of the microporous media can be controlled by adjusting the electrical signal that is applied to the electrodes. For example, dendrites can form picket fence type structures or fractal type structures.

A method for forming microporous media on an APEX device could involve forming a long channel with opposing metal electrodes. When this channel is filled with the appropriate chemical and an appropriate electrical signal is applied to the electrodes, dendrites will form in the interstitial space between the electrodes forming a microporous media.

Returning to Fig. 6, an electronic reagent dispenser system 74 may be provided to deliver reagents to the system 60. Preferably, the reagents are delivered by electrophoretic force if they are charged. Optionally, an electronic waste disposal system 76 is included within the system 60. The waste disposal system 76 attracts charged waste particles to it and disposes of them by holding the charged entities on it. Another optional member of system 60 is the DNA fragment storage system 78. This fragment storage system 78 serves to temporarilly hold DNA fragments for future analysis..

The system 60 may include some or all of the functions described above. For example, the combination of sample preparation in the form of complexity reduction, as performed by the DNA selector 68 and restriction fragment selector 70 may be associated with the analyti-

cal matrix 72. However, any or all of the above described functions may be combined as desired.

Fig. 7 shows a block diagram of the overall system including the controller. The underlying electrodes in an APEX device are made active by the application of a controlled potential to the electrode or by the sourcing of a controlled current through the electrode. Full functionality is realized when the potential or current at each electrode of the APEX device is independently controlled. This is accomplished by an APEX controller system.

The controller computer 80 interfaces with user input/output devices, such as a display 82 and input device 84. The display 82 may be any form of conventional display such as a monitor or computer screen. The input 84 may be any conventional user input device, such as a keyboard, mouse, or touch-screen device. The controller computer 80 is connected with the power supply and waveform generator 86. The controller 80 sets the power supply and waveform generator 86 to provide the current or voltage output to the interface 88. In the preferred embodiment, the power supply or waveform generator 86 is capable of providing precisely regulated and voltage and current sourcing. The controller computer 80 provides control signals to the interface 88 via the multifunction input/output board 90. The interface 88 provides a simplified connection to the contacts for the APEX system 92.

The interface preferably includes relays that permit selective connection between the power supply and waveform generator 86 to the specific electrodes of the APEX system 92. In one embodiment, the interface 88 comprises a plurality of relays which connect the power supply and waveform generator 86 to the APEX system 92 electrodes. The connections permit the selection or non-selection of a path between the power supply and waveform generator 86 to the APEX system 92 electrodes.

Additionally, another relay permits selecting the polarity of the voltages supplied to the APEX system 92 electrodes. Optionally, if multiple source levels are available, such as from a multiple output power supply 86, the specific level to be connected to an APEX system 92 electrode may be set independently of those for the other electrodes.

Thus, as described in connection with Fig. 2A, by placing certain electrodes (e.g., 12B and 12C) at a negative, but lesser potential than electrode 12D, the attachment region 16B and 16C would be protected by the local force field.

The interface 88 may serve to select the desired voltage for the individual electrodes in the APEX system 92. Alternatively, such a different voltage arrangement may be achieved through use of a voltage divider.

In the preferred embodiment, the controller computer 80 is a Macintosh Quadra 950. National Instruments Corporation LabVIEW software is used to provide a software interface for a user to program the devices connected to the APEX and to collect and process data from an assay. National Instruments NuBus boards are used to provide the hardware interface from the Quadra 950 computer 80 to the power supply devices 86 that source potentials and currents and that measure the actual currents and potentials and the results of the assay.

The user controls the assay through a Virtual Instrument created with the LabVIEW software. The virtual instrument provides a user friendly graphical representation of the controls that the user may exercise, and of some of the results of applying these controls to the APEX device to perform an assay. The user interfaces with the Virtual Instrument through the keyboard and mouse (collectively, input 84) of the Quadra 950 computer 80. The Virtual Instrument provides software interfaces to a National Instruments NB-MIO-16XL multipurpose input/output 90 and to a National Instruments DMA2800

board that are connected to the NuBus data bus of the Quadra 950.

The multipurpose I/O board is able to provide digital and/or analog signals to external devices to implement the programmed sequence specified by the user through the Virtual Instrument. The MIO board is also able to digitize and store in the Quadra 950, under control of the Virtual Instrument, signals generated by the devices connected to the APEX. The DMA2800 provides the ability to store rapidly the data acquired by the MIO board through Direct Memory Access, bypassing the Quadra 950 CPU. The DMA 2800 also provides a GPIB (IEEE 488) interface for control of external devices that adhere to the IEEE 488 communication and data transfer standard, which includes most modern instruments.

In this preferred embodiment of the controller, two external devices are used to source the potentials or currents to the APEX. A Keithley 236 Source/Measure Unit power supply 86 provides adequate stability and flexibility as a source of precisely regulated potential or current. The SMU 236 either applies a potential and measures the resultant current or provides a source of current and measures the resultant potential. This device is programmed from the Virtual Instrument under GPIB control through the DMA2800 board to control the current or potential levels and time dependence, and to measure and store the actual potentials and currents that are sourced to the APEX.

The sourced currents or potentials are applied to the APEX through an array of relays in interference 88 that provide independent switching of each electrode between no connection, connection to positive source and connection to negative source. The preferred embodiment also provides for more than one Source/Measure supply to be utilized to provide different levels of positive and negative potential or current to different electrodes. The array of relays is provided by a National Instru-

ments SCXI Chassis with nine 16-channel, Class 3 Relay Modules connected in the chassis, providing a total of 144 relays. Two relays are used per electrode to provide for electrode disconnected or electrode connected to either positive or negative source. In the preferred embodiment, a bundle of cables connects these relays to the APEX device through a Cerprobe Probe Card that provides mechanical contact of probes to the bond pads of the APEX device.

The controller computer 80 optionally controls the illumination source 94 for excitation of fluorescence to detect DNA hybridization. In the preferred embodiment, the illumination source 94 is a laser which outputs radiation at an appropriate wavelength to excite fluorescent markers included within the APEX system 92.

The output of the APEX system 92 is passed through observation path 96 to the detector 98. The observation path 96 may be a physical connection, such as through a fiber optic, or may comprise an optical path such as through a microscope. Optical filters may be utilized in the observation path to reduce illumination of the detector at wavelengths not corresponding to the emission spectra of the fluorescent markers in the APEX system 92. Additionally, notch filters may be utilized as necessary to reduce illumination of the detector 98 at the excitation wavelength of the laser illumination source 94. The detector 98 may optionally form an image of the APEX system 92, such as through the use of a cooled CCD camera. In addition to, or as an alterative to, forming an optical image, the emitted fluorescence radiation from the APEX system 92 may be detected by conventional means such as photodiodes or photomultiplier tubes. The output of the detector 98 is provided to the data processing/analysis system 100. This system monitors the level of detected probe material in the APEX system 92. Optionally, an expert system may be utilized in the analysis system 100.

In the preferred embodiment, a Data Translation Frame Grabber board is interfaced to the Quadra 950 NuBus, to provide capture to memory of images recorded by video cameras such as the Optronics cooled color CCD camera used in the preferred embodiment. This CCD camera observes the APEX device through a microscope with appropriate filters to provide visualization of fluorescence on the APEX array.

Alternate systems may implement all the functionality of the controller as described, but may use custom devices incorporated into printed circuit boards and custom software to control the board with a similar user-friendly interface for programming the device. These alternate systems may also incorporate the switching elements of the array of relays into a semiconductor device underlying the active, programmable matrix system. Figure 8 shows a cross-sectional view of an alternative embodiment for the active, programmable matrix system. Individually addressable electrodes 102 are formed upon a support layer 104. Preferably, the support layer 104 is an insulator. Above the electrodes 102 is preferably disposed permeation layer 106 and individual attachment layers 108 corresponding to the individual electrodes 102. Electrical connections 110 are provided from the backside of the electrodes 102 through the support 104. Additionally, a semiconductor support 112 includes circuit elements 114 connected to the conductor 110. The circuit elements 114 may be formed on or in the semiconductor 112. The circuit elements 114 may provide individual control of the voltage and or current provided via the conductor 110 to the electrode 102. In particular, the circuit elements 114 may incorporate the switching elements of the array of relays described in the preferred embodiment. Multiple current/voltage source lines 116 to each circuit element 114 provide the capability to source different levels to different electrodes 102. Memory type address lines 118

provide convenient activation paths for the individual circuit elements 114.

Waveguides can be used for guiding excitation light to micro-locations, and for guiding fluorescence signals to detectors. Waveguides can be free standing, as in an optical fiber, or can be integrated into a monolithic semiconductor device. Waveguides can be fabricated from materials such as zinc oxide or indium tin oxide that are also electrically conductive. The waveguide can then serve as both an electrode and as means for transporting optical radiation. Waveguides can be located in or around the plane of the capture probe to minimize nonspecific background fluorescence. Waveguides can incorporate holographic optical elements. The function of these holographic optical elements includes, but is not exclusive to, notch filters, dichroic mirrors, band pass filters, beam splitters, neutral density filters, half-wave plates, quarter-wave plates, polarizers, and lenses.

Fig. 9 shows in cross-section an alternative, layered structure for the active programmable matrix system. In a first layer, individual electrodes 120 are formed upon a support 122. The support 122 is preferably insulating. Above the electrodes 120 is preferably formed a permeation layer 124 and individual attachment layers 126 corresponding to the individual electrodes 120. Optical paths 128 are provided through the support 122 to access the electrode 120. Preferably, the optical path 128 is comprised of a fiber optic or light guiding pipe or structure. Optionally, electrical connection 130 passes through the support 122 to access the electrodes 120 from the backside. The term backside is used herein to connote that side of the electrode 120 which contacts the support 122. In a second layer a semiconductor support 132 includes circuit elements 133 connected to the conductor 131. The conductor 131 is designed such that its upper surface mates and forms

good electrical contact with the bottom of the conductor 130 on the backside of the first layer. The circuit elements 133 may be formed on or in the semiconductor 132. The circuit elements 133 may provide individual control of the voltage and or current provided via the mated conductors 131 and 130 to the electrode 120. In particular, the circuit elements 133 may incorporate the switching elements of the array of relays described in the preferred embodiment. These circuit elements may be supplied by multiple current/voltage source lines and may be activated by memory type address lines. Optionally, detector elements 134, such as photodiodes may be incorporated in the semiconductor layer 132 and coupled through optical paths 135 with the optical paths 128 of the first layer, so that the detector elements monitor DNA hybridization at the attachment sites 126 of the first layer. These optical paths can implemented as fiber optic paths or as waveguides and can incorporate various optical elements as described above. Optionally, a sample containment vessel 136 may be disposed around the structure to contain the biological material under analysis or test. Optionally, fluid input ports 137 or optical viewing ports 138 may be provided. The biological containment structure 136 and optional port 137 and 138 may be used in connection with any of the active, programmable matrix systems described herein.

Fig. 10 shows a perspective view of a mounting system for an active, programmable matrix system. A system 140 may be formed on a chip using a structure such as that shown in Figs. 3 and 4. The chip 140 is connected via bonding wires 146 between the contact pads (Fig. 3) and the chip carrier 144 connection pads 142. The chip carrier 144 preferably includes individual pins 147 which provide electrical connection via the pads 142 to the bonding wire 146 onto the chip 140. The pins 147 mate with receptacle 148 which is in turn connected to the control system.

Active, programmable matrices of micro-locations can also be formed from capillary tubes. Fig. 17 shows a system and product formed therefrom. Capillary tube matrices are formed by stacking capillary tubes in arrays 220 of arbitrary geometry, or by melting by heater 222 and drawing, such as by die 224, these arrays 220 into an adherent and integral unit 226. Alternatively, solid rods composed of two diffenent materials arranged about each other concentrically can be used instead of capillary tubes. Fig. 18 shows such a structure in perspective. Here, the material that composes the inner core 230 is etched out from the outer material 234 selectively to form a hole 232 that goes partially or all the way through the device. Alternatively, the inner core may be etched in such a way as to form a controlled porousity glass.

Individual capillary tubes can be addressed by wires inserted into the capillary tube, or by affixing the capillary tube matrix to a complimentary matrix of lithographically formed electrodes. Additionally, the inner cores of the solid rods may be formed from a conducting material. Electrical contact can be made with the inner core material by affixing the solid rod matrix to a complimentary matrix of electrodes, or by lithographically forming electrodes on the solid rod matrix.

The capillary tubes and etched solid rods are filled with an appropriate material to form a permeation layer. The surface of the permeation layer can be functionalized with specialized attachment chemistry.

An alternative method to electrophoretic transport is to use convective mass transport to transport material to microlocations. One device that can accomplish this is a rotating disk. Here convection is acheived by the hydrodynamic shear forces present at boundary between the spinning disk and the solution. Fluid flows straight onto the surface from the bulk solution. A matrix of electrode pads can be attached to a spinning

disk, or each electrode in the matrix can be attached to a separate disk. In the latter case, each electrode can be addressed selectively by convective mass transport. After the pads are addressed by convective mass transport, the electrode can be used to remove unwanted material using electrophoretic transport.

A preferred process for forming an active, programmable matrix system is described in Figs. 11A-G. A semiconductor 150, preferably p-type, test grade silicon, has a thick (10,000 Å) oxide 152 formed upon it. Fig. 11B shows a metal layer 154 formed on the oxide layer 152. Preferably, the metal is chosen from the group consisting of: aluminum, gold, platinum, paladium, titanium, titanium/tungsten. Semiconducting polysilicon may also be used in place of the metal. Fig. 11C shows patterned aluminum 156 formed upon the oxide layer 152. The metal may be patterned by any conventional lithographic technique, such as photolithography.

Fig. 11D shows the structure of Fig. 11C with an overcoat of glass, such as TEOS. The TEOS is preferably formed by PECVD techniques. Preferably, the glass is formed at a relatively high temperature, such as 475°C to promote adhesion to the metal layer 156. The TEOS layer 158 is then overcoated with a nitride layer 160. The nitride layer 160 and TEOS layer 158 are preferably etched in the region above the patterned electrode region 156. This forms a window 162 permitting direct contact to the patterned electrode 156.

Fig. 11G shows the result of exposing the overall structure to aminopropylsilene (APS). The APS 164 adheres to the patterned metal layer 156 and not to the nitride layer 160. The APS layer serves as the attachment layer for DNA capture probes.

Fig. 12 shows an alternative structure in which polysilicon is used in lieu of normal metal contacts. The structure is similar to that of Fig. 11F but includes a polysilicon layer 166 in lieu of the aluminum

layer 156. The sequence of preferred steps is as follows. First, the semiconductor, preferably p-type, test grade silicon is oxidized with a thick (10,000 Å) oxide. A conductive polysilicon layer, preferably a polysilicon doped 5,000 Å thick layer is formed. The polysilicon is then patterned, preferably by photolithography using a wet etch. Next, a glass layer, such as PECVD deposited TEOS is formed. A layer approximately 3,000 Å formed at 475°C is preferred for improved adhesion. The glass layer is then patterned, again, preferably with photolithographic techniques using a wet etch. A metal layer is then formed over the surface, preferably by sputtering aluminum to a thickness of 3,000 Å. The metal is then patterned, again preferably photolithographically, with a wet etch. Next, a nitride layer is formed, preferably via PECVD at 70°C to a thickness of 3,000 Å. Next a via is formed photolithographically using a wet etch so as to contact the electrode.

Fig. 13 shows a structure having improved adhesion of metal conductor to underlying insulating layers through the use of an intermediate adhesive metal such as titanium tungsten. A semiconductor 170, preferably silicon, has disposed thereon an oxide layer 172. An intermediate electrode layer 176, formed of a conductive metal such as gold or aluminum, is sandwiched between titanium tungsten 174 and 178. Adhesive metal layer 178 contacts the external electrode 184, preferably formed of platinum. A glass layer 180, such as formed from TEOS, underlies a external nitride coating 182.

Fig. 14 shows a cross-sectional view of an improved electrode arrangement. An electrode 190 is disposed adjacent to an insulator 192, preferably an oxide. A nitride layer 194 overlies the insulator layer 192. Preferably, the nitride layer 194 is undercut such that the insulating layer 192 is set back from the edge 198 of the nitride layer. A reservoir 196 is thereby de-

fined, having a larger volume than a similar structure without the undercut region.

The structure illustrated in Fig. 14 is used to hold mechanically a plug of material that forms a permeation layer. The overhanging layer captures the permeation layer. This design can be generalized to an arbitrary number of overhanging layers, so as to form an arrangement such as a beehive shape of decreasing concentric circles, or to have varying radii of concentric disk apertures.

The permeation layer (e.g., layer 14 of Fig.2) may be formed from materials such as, but not exclusive to, carbon chain polymers, carbon-silicon chain polymers, carbon-phosphorous chain polymers, carbon-nitrogen chain polymers, silicon chain polymers, polymer alloys, layered polymer composites, interpenetrating polymer materials, ceramics, controlled porousity glass, materials formed as sol-gels, materials formed as aero-gels, materials formed as hydro-gels, porous graphite, clays or zeolites.

Permeation layers separate the binding entities from the surface of the electrode. Micro-locations have been created using microlithographic and micro-machining techniques. Chemical modification of the surface of the micro-locations and of polymer layers over the micro-locations have been used to create specialized attachment sites for surface functionality.

Mesh type permeation layers involve random arrangements of polymeric molecules that form mesh like structures having an average pore size determined by the extent of cross-linking. We have demonstrated the formation of mesh type permeation layers using several polymerizable formulations containing acrylamide as a monomer. We have used triethylene glycol diacrylate, tetraethylene glycol diacrylate and N, N'-Methylene-bis-acrylamide as cross-linking agents. Poly-l-lysine with molecular weights of 330 kilodaltons and 25 kilodaltons

was mixed into the acrylamide/copolymer formulation to provide a means for attaching specialized functionality to the surface of the permeation layer. The mixture was cast onto the surface of the micro-location. It was then photopolymerized by ultraviolet light. In some cases, AuCl4 was added as a photoinitiator. The polymer formulations were cast from water and the nonaqueous solvents, methanol, tetrahydrofuran, acetonitrile, acetone, and mixtures of these solvents.

DNA capture probe was attached to the surface of the permeation layer by a Schiff base reaction between an oxidized ribonucleoside attached to the DNA capture probe and the primary amine of the poly-l-lysine. This provides evidence of covalent attachment of special functionality to the surface of the permeation layer.

An oxidized DNA capture probe was brought to a surface micro-location by electrophoretic transport. The capture probe was labeled with a fluorescent marker. This demonstrates the ability to address a micro-location by electrophoretic transport.

An oxidized capture probe with a fluorescent marker attached was attracted to the surface of the permeation layer at a micro-location by electrophoretic transport. The permeation layer was removed from the micro-location by mechanical means. No evidence of the presence of the fluorescently labeled capture probe was observed. This demonstrates the ability of the permeation layer to protect the DNA from the electrode surface.

The maximum DC current density that was attained at a gold micro-location, which was not modified with a permeation layer, before bubbles due to water hydrolysis appeared was 8 milliampheres/cm2. The maximum DC current density that was attained at a gold micro-location, which was modified by an acrylamide-based permeation layer, before bubbles due to water hydrolysis appear was 40 milliampheres/cm2. This demonstrates the ability of the permeation layer to raise the maximum accessible

current density before bubbles form due to water hydrolysis.

An ionomer sandwich permeation layer is formed from one or more lamina of polyelectrolytes. The polyelectrolyte layers may have the same charge, different charge, or may be charge mosaic structures.

A two layer ionomer sandwich layer was formed from a base layer of a perfluorinated sulfonic acid polyelectrolyte (Nafion) and an upper layer of poly-l-lysine. The base Nafion layer was cast onto a micro-location and allowed to dry. This base layer was then exposed to a 1% by weight aqueous solution of poly-l-lysine. The cationic lysine-based polymer adsorbed strongly to the anionic Nafion base layer. The poly-l-lysine layer allowed the attachment of an oxidized DNA capture probe to the surface of the permeation layer by a Schiff base reaction. The Nafion base layer is anionic and is permselective toward negative ions such as DNA.

Fig. 15 shows examples of the graphical user interface. Window 200 shows an overall view of the display. Identification information 202 is provided. The various pads of the active, programmable matrix system are identified in a rectangular coordinate system. The displays 204 each show the electrical parameter, such as current or voltage for particular pads. Box 204A shows the current as a function of time for a pad, (3,4), wherein the current varies as a function of time, changing directions during the course of the application. Box 204B shows a pad, (3,5), having no applied current during the time shown. Box 204C shows a time varying current for pad (4,4), wherein that current is delayed with respect to time relative to the pad (3,4) reported in Box 204A. Box 204D shows a pad, (4,5), with no applied current as a function of time. Box 204E shows a pad, (1,1), for which the voltage has a constant, negative DC value. Box 204F shows the voltage as a function of time for a pad, (3,4) having a more negative DC value. In all

cases, the boxes show the programmed current or voltage as a dotted line, and the measured current or voltage as a solid line.

In addition to the preferred embodiment of the invention and the alternatives described above, several more alternatives are possible. For example, the electric field that gives rise to ion migration may be modulated in time as long as a DC bias voltage or current is applied simultaneously. The use of an AC signal superimposed on a DC bias voltage or current can achieve three things, 1) minimize the background due to nonspecifically bound DNA, 2) provide a means of electronic stringency control where the control variable is the frequency of the alternating current or voltage, 3) provide a means of aligning DNA molecules spatially.

Many alternatives to the detection of hybridized DNA by fluorescence exist. Most of the alternative techniques also involve modification of capture or target or reporter DNA probes with reporter groups that produce a detectable signal. A few of these techniques based on purely physical measurements do not require reporter groups. These alternative techniques are catalogued as follows: (1) Linear Optical Methods including fluorescence, time modulated fluorescence, fluorescence quenching modulation, polarization selective fluorescence, absorption, specular reflectance, changes in index of refraction, ellipsometry, surface plasmon resonance detection, chemiluminescence, speckle interferometry and magneto-optic Kerr effect; (2) Nonlinear Optical Methods including second harmonic generation, third harmonic generation, parametric mixing, optical heterodyne detection, phase conjugation, soliton damping and optical Kerr effect; (3) Methods Based on Thermal Effects including differential scanning calorimetry, multifrequency differential scanning calorimetry, and differential thermal analysis; (4) Methods Based on Mass Changes including crystal microbalances, cantilever

microbalances, surface acoustic waves and surface Love waves; (5) Electrochemical Methids including amperometry, coulometry, voltammetry, electrochemiluminescence, charge transfer in donor-acceptor complexes and surface impedance spectroscopy; and (6) Radioactivity Detection Methods using labeled groups.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Claims:

1. An electronic device adapted for performing molecular biological processes comprising:

a support having a first generally planar surface,

a plurality of self-addressable electrodes disposed on the first surface of the support, the electrodes having a contacting portion adjacent the first surface of the support, and

individual electrical connections to the electrodes.

2. The electronic device of Claim 1 wherein the individual electrical connections to the electrodes comprise leads disposed on the first surface of the support.

3. The electronic device of Claim 1 wherein the individual electrical connections to the electrodes are electrical pathways extending from the contacting portion of the electrodes into the support.

4. The electronic device of Claim 1 wherein the support includes an insulator.

5. The electronic device of Claim 4 wherein the insulator is an oxide.

6. The electronic device of Claim 1 wherein the support includes a semiconductive material.

7. The electronic device of Claim 6 wherein the support including the semiconductor further includes an oxide layer disposed thereon.

8. The electronic device of Claim 6 wherein control electronics are included within the semiconductive

material and connected via the individual electrical connections to the electrodes.

9. The electronic device of Claim 1 further including an optical access to the electrodes.

10. The electronic device of Claim 9 wherein the optical access is formed through the support so as to access the contacting portion of the electrodes.

11. The electronic device of Claim 9 or 10 wherein the optical access includes a fiber optic.

12. The electronic device of Claim 9 or 10 wherein the optical access includes a light pipe.

13. The electronic device of Claim 9 wherein the access comprises an opening.

14. The electronic device of Claim 1 further including a permeation layer disposed upon the electrodes.

15. The electronic device of Claim 1 further including an attachment layer disposed above the electrodes.

16. The electronic device of Claim 14 further including an attachment layer disposed above the permeation layer.

17. The electronic device of Claim 1 further including a containment vessel disposed above the support.

18. The control system for use with an active, programmable electronic microbiology system having a plurality of electrodes comprising:

a controller adapted to receive user input and provide output including control signals to generate independent electronic environments at the electrodes,

a input system for receiving user instructions connected to the input of the controller,

a generator to provide the desired electronic environment at the electrode, the generator operating under control of the controller output, and

an interface adapted to connect the control system to the active, programmable electronic system.

19. The control system of Claim 18 wherein the generator comprises a power supply.

20. The control system of Claim 19 wherein the power supply is a regulated power supply.

21. The control system of Claim 18 wherein the generator comprises a waveform generator.

22. The control system of Claim 18 wherein the interface comprises a relay system.

23. The control system of Claim 22 wherein the interface system includes relays to provide selective connection from the generator to the electrodes.

24. The control system of Claim 22 wherein the interface includes relays adapted to change the polarity of the output of the generator.

25. The control system of Claim 22 wherein the interface includes relays to provide selective connection to either a first level or second level of a signal.

26. The control system of Claim 25 wherein the signal is a predetermined voltage level.

27. The control system of Claim 25 wherein the signal comprises current at a predetermined level.

28. The control system of Claim 18 wherein the controller comprises a computer.

29. The control system of Claim 18 wherein the controller is microprocessor based.

30. The control system of Claim 18 further including an output system for providing information to the user.

31. The control system of Claim 30 wherein the output system includes a display.

32. A method for complexity reduction in a solution including DNA comprising the steps of:

binding the DNA to a support, leaving unbound at least a portion of the DNA containing desired material,

severing the bound material from the unbound material, and

removing the severed material from the remaining bound material.

33. The method for complexity reduction in a solution containing DNA of Claim 32 wherein the binding is performed by hybridizing the DNA with complimentary DNA.

34. The method of complexity reduction of Claim 32 wherein the severing is achieved through use of restriction enzymes.

35. The method of complexity reduction of in a solution containing DNA Claim 32 wherein the severed material is removed through electrophoretic force.

36. A system for performing molecular biology reactions comprising:

an input for receiving a sample containing material to be analyzed,

a sample preparation unit, and

an active, programmable electronic device including a plurality of separately addressable electrodes.

37. The system of Claim 36 wherein the active, programmable matrix comprises an array of electrodes.

38. The system of Claim 36 wherein the active, programmable electrodes further include an attachment layer disposed above the electrodes.

39. The system of Claim 38 wherein the attachment layer includes capture sequences.

40. The system of Claim 36 further including a detector operatively positioned to monitor the active, programmable device.

41. The system of Claim 40 wherein the detector comprises an imaging system.

42. The system of Claim 41 wherein the imaging system comprises a CCD camera.

43. The system of Claim 40 further comprising an analysis system adapted to receive the output of the detection system.

44. The system of Claim 36 wherein the sample preparation unit includes a cell sorter.

45. The system of Claim 36 wherein the sample preparation unit includes a DNA selector.

46. The system of Claim 36 wherein the sample preparation unit includes a restriction fragment selector.

47. The system of Claim 36 wherein the sample preparation unit includes an amplification system.

48. The system of Claim 47 wherein the amplification unit utilizes polymerase chain reaction.

49. The system of Claim 36 further including a reagent delivery system.

50. The system of Claim 49 wherein the reagent delivery system is an electronic reagent delivery system.

51. The system of Claim 49 wherein the reagent delivery system is a fluidic reagent delivery system.

52. The system of Claim 36 further including a waste disposal system.

53. The system of Claim 52 wherein the waste disposal system is an electronic waste disposal system.

54. The system of Claim 52 wherein the waste disposal system is a fluidic waste disposal system.

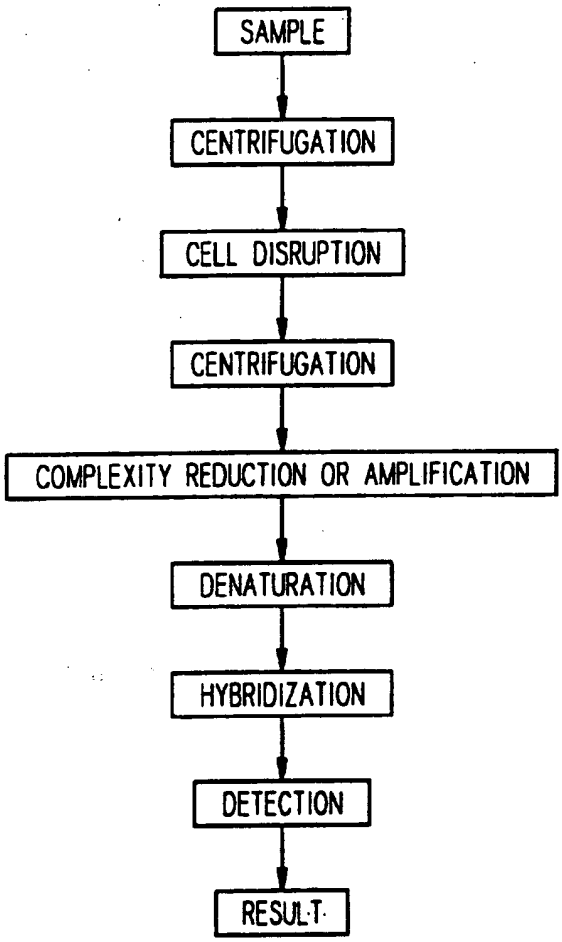

SAMPLE
CENTRIFUGATION
CELL DISRUPTION
CENTRIFUGATION
COMPLEXITY REDUCTION OR AMPLIFICATION
DENATURATION
HYBRIDIZATION
DETECTION
RESULT

FIG. 1

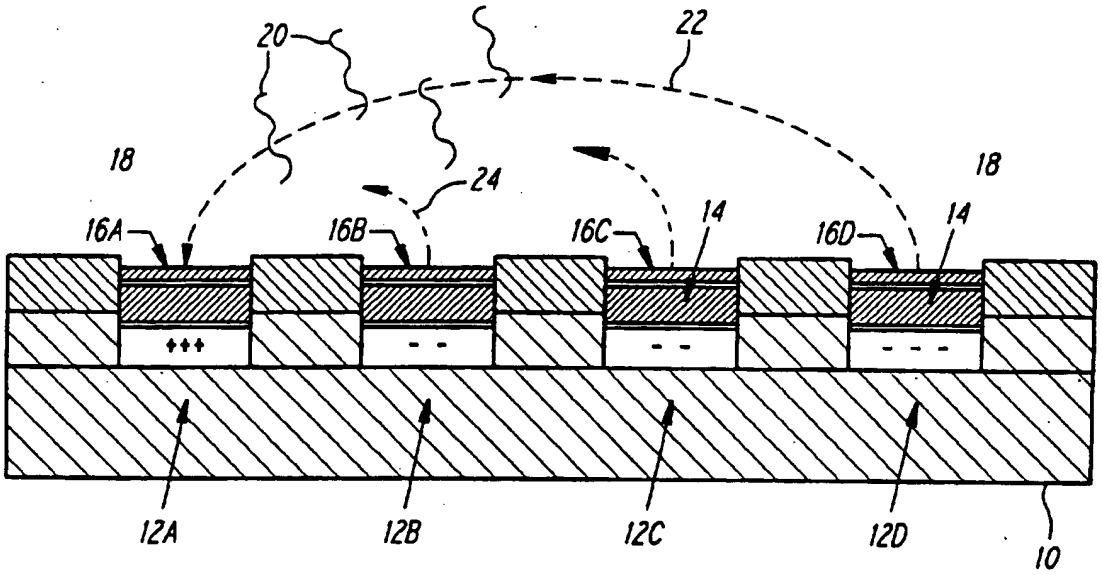

20
22
18
24
18
16A
16B
16C
14
16D
14
12A
12B
12C
12D
10

FIG. 2A

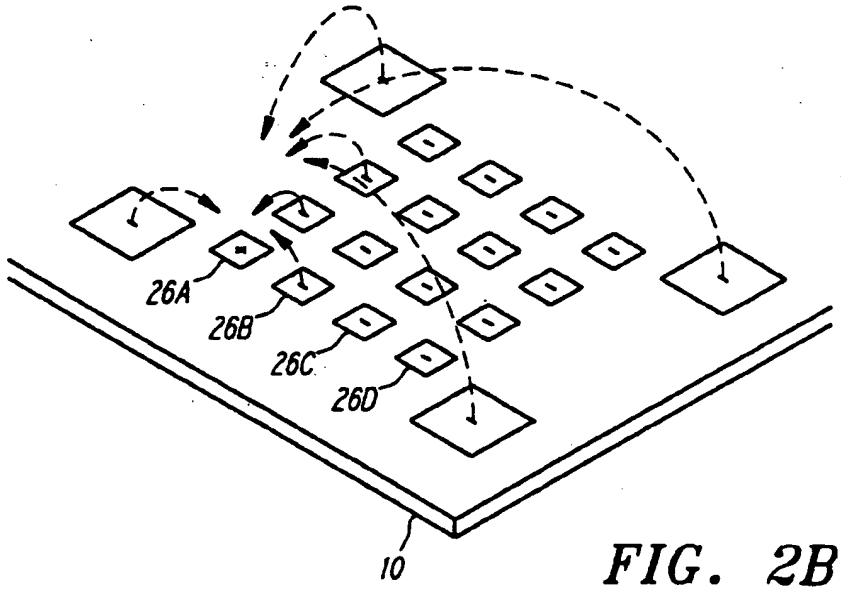

26A
26B
26C
26D
10

FIG. 2B

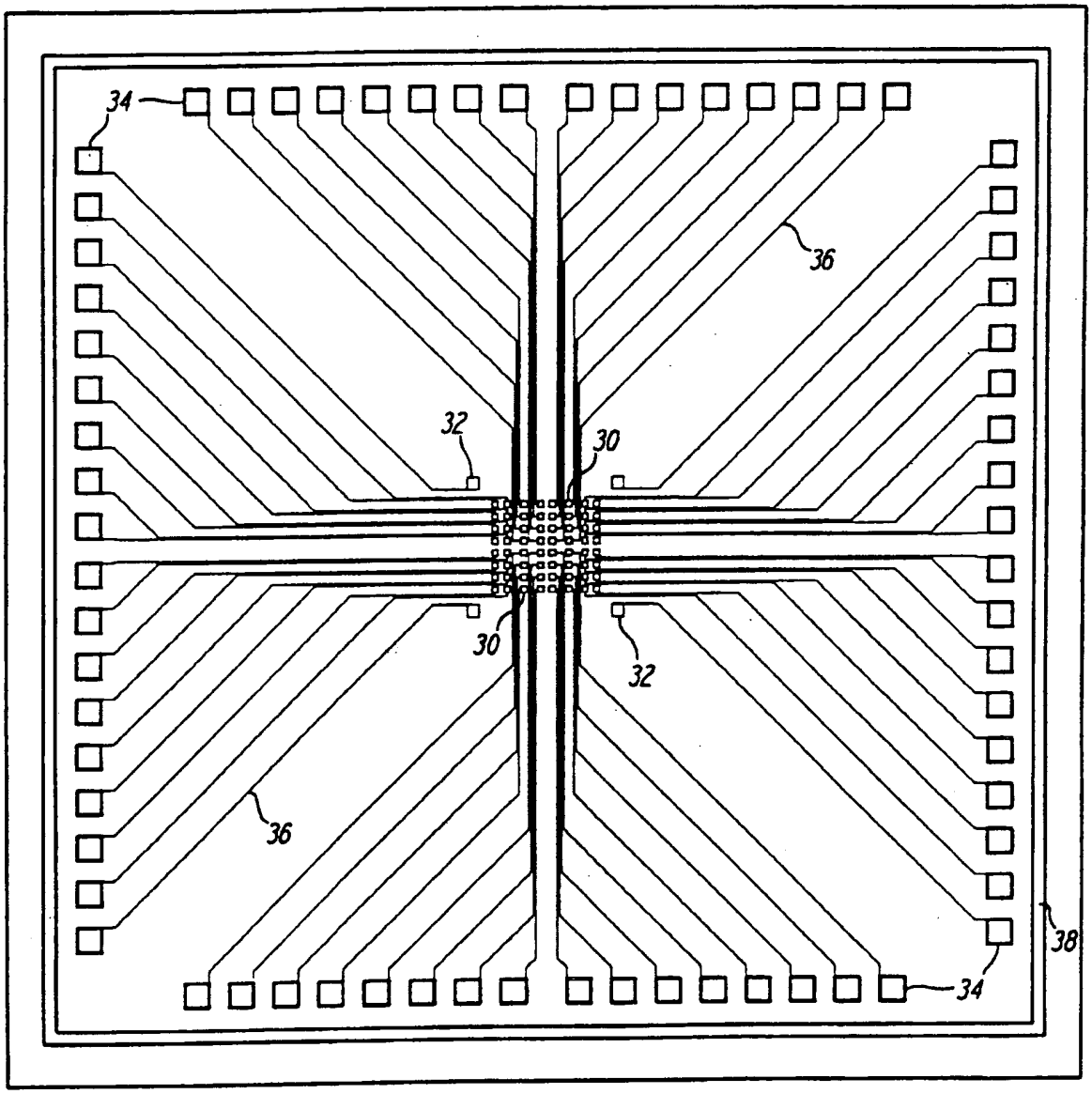


FIG. 3

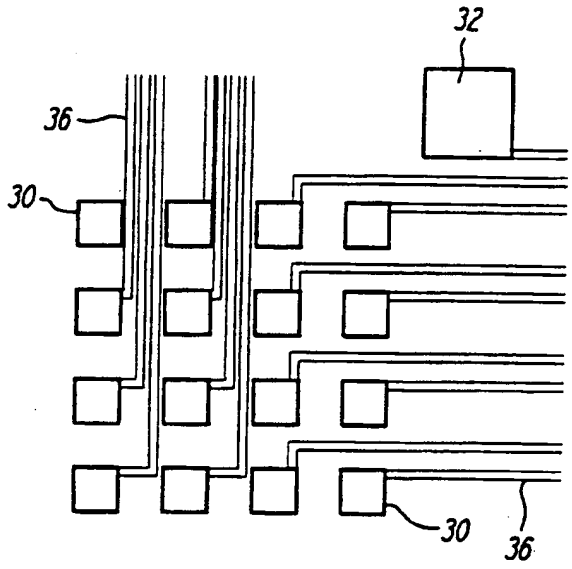


FIG. 4

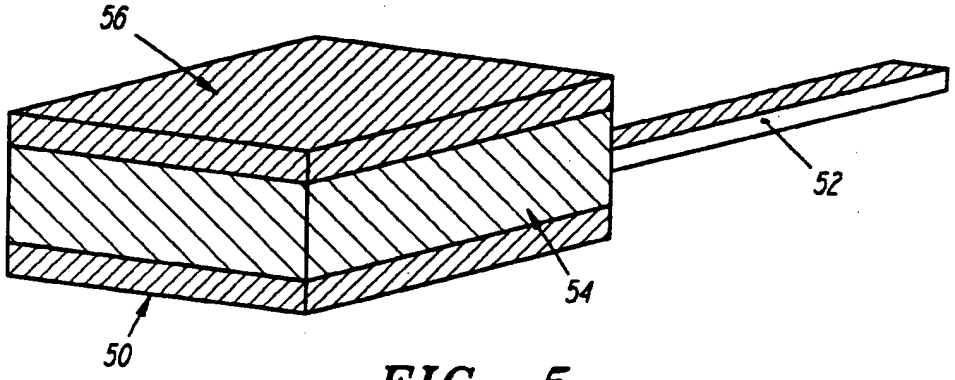


FIG. 5

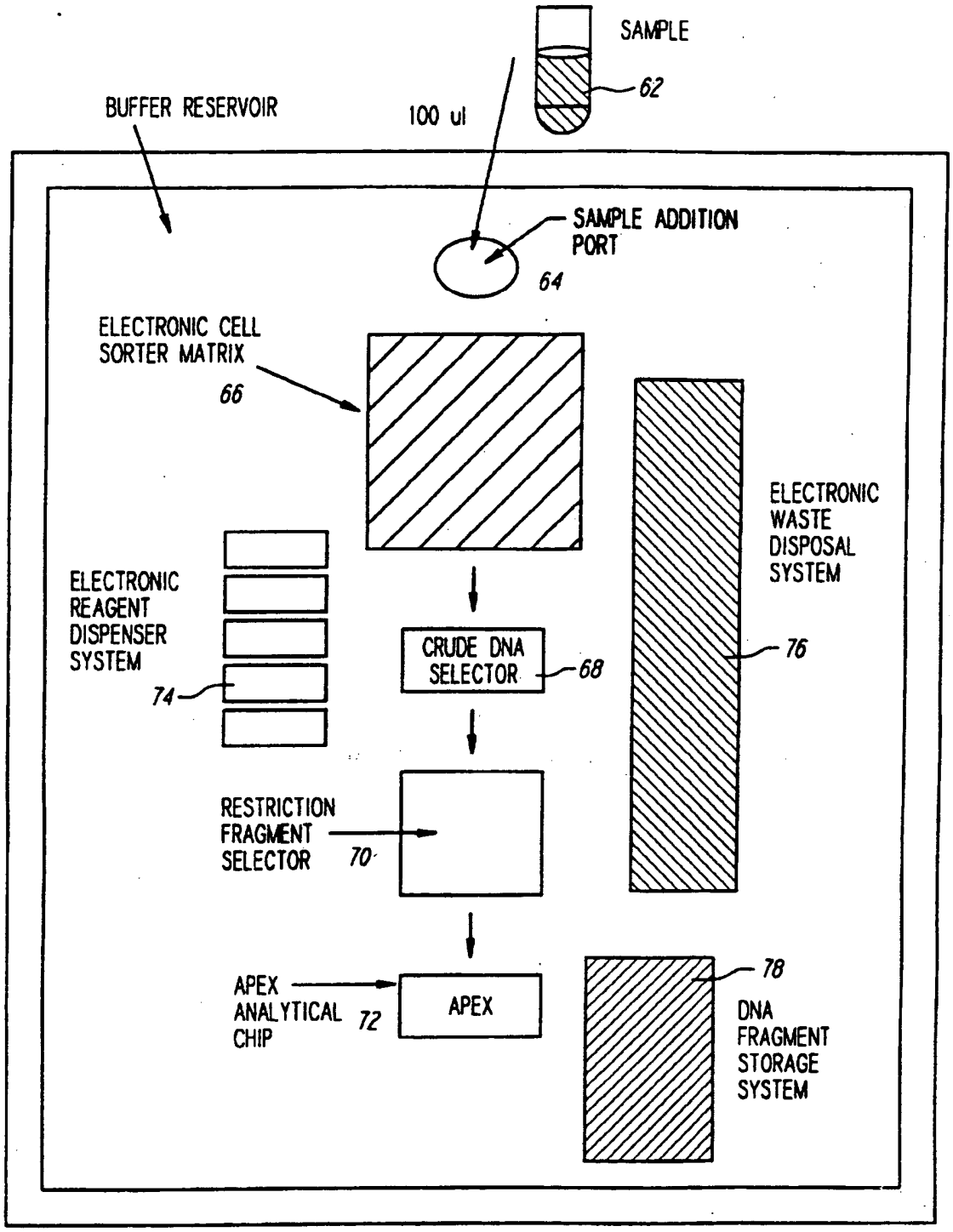

SAMPLE
62
BUFFER RESERVOIR
100 ul
SAMPLE ADDITION PORT
64
ELECTRONIC CELL SORTER MATRIX
66
ELECTRONIC WASTE DISPOSAL SYSTEM
76
ELECTRONIC REAGENT DISPENSER SYSTEM
74
CRUDE DNA SELECTOR
68
RESTRICTION FRAGMENT SELECTOR
70
APEX ANALYTICAL CHIP
72
APEX
78
DNA FRAGMENT STORAGE SYSTEM

FIG. 6

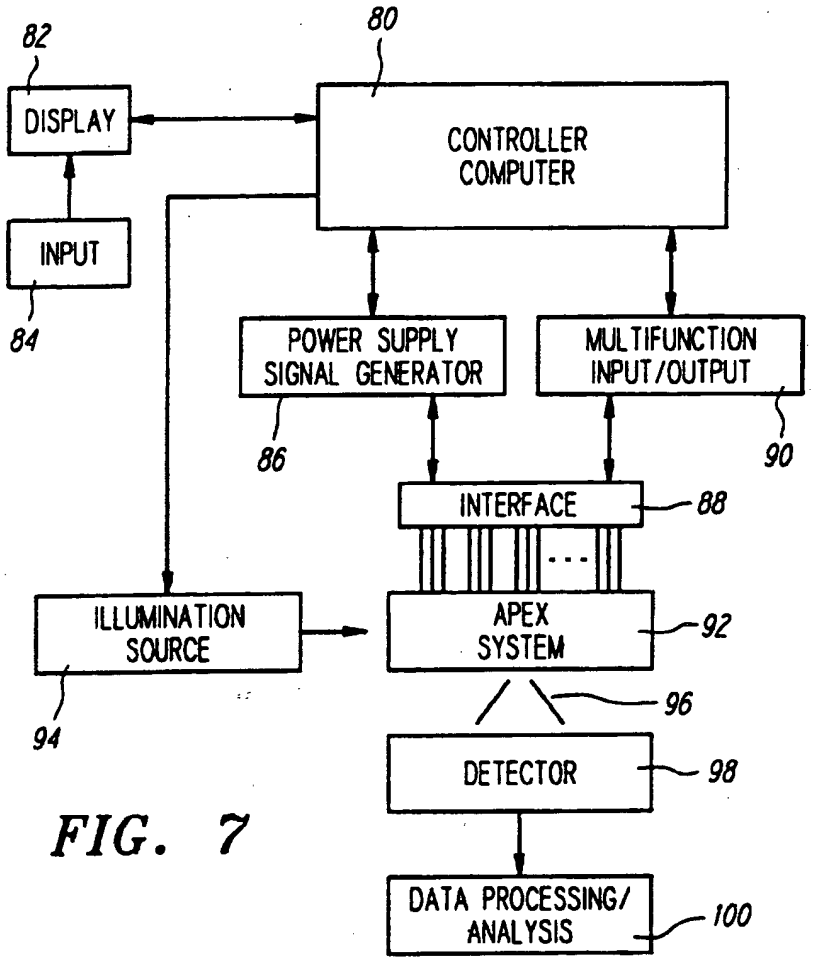

82
80
DISPLAY
CONTROLLER COMPUTER
INPUT
84
POWER SUPPLY SIGNAL GENERATOR
MULTIFUNCTION INPUT/OUTPUT
86
90
INTERFACE
88
ILLUMINATION SOURCE
APEX SYSTEM
92
96
94
DETECTOR
98
DATA PROCESSING/ ANALYSIS
100

FIG. 7

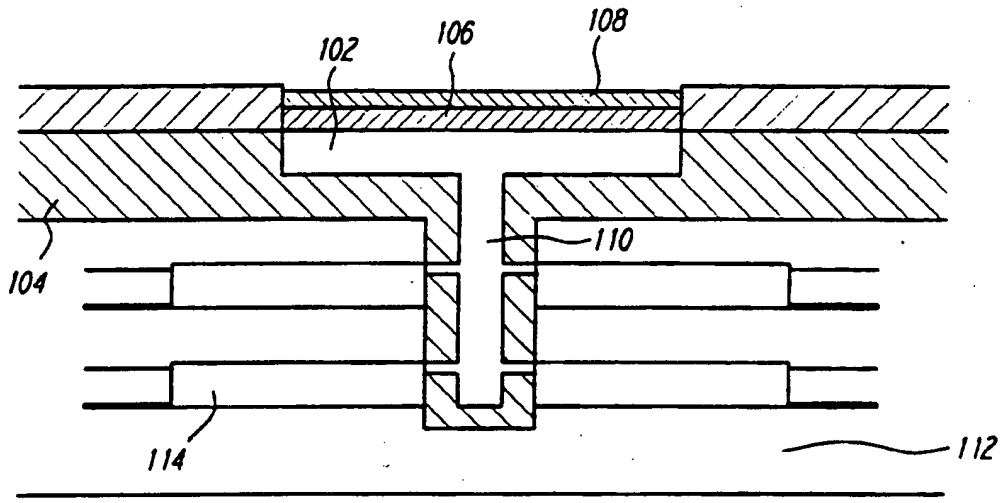


FIG. 8

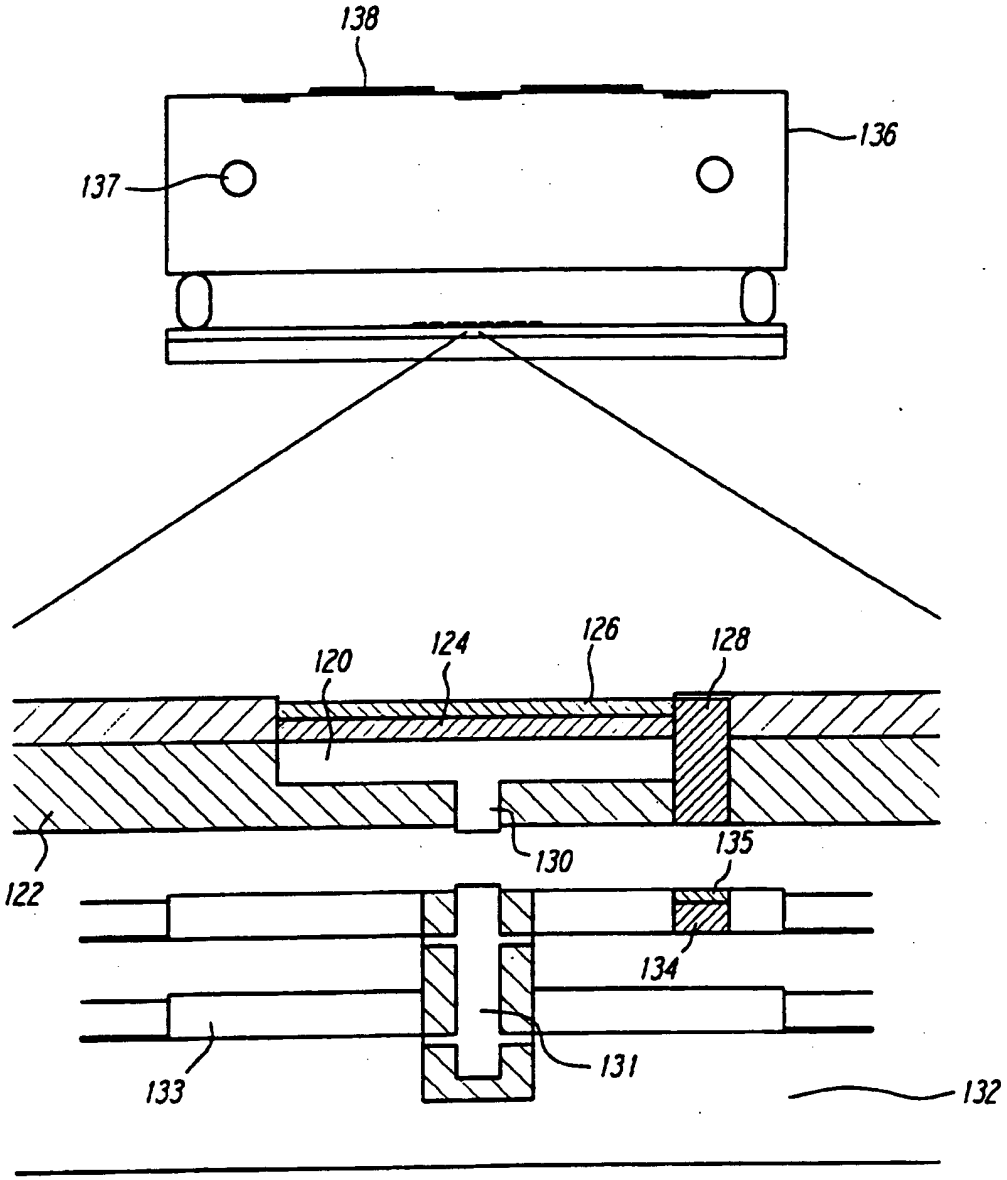


FIG. 9

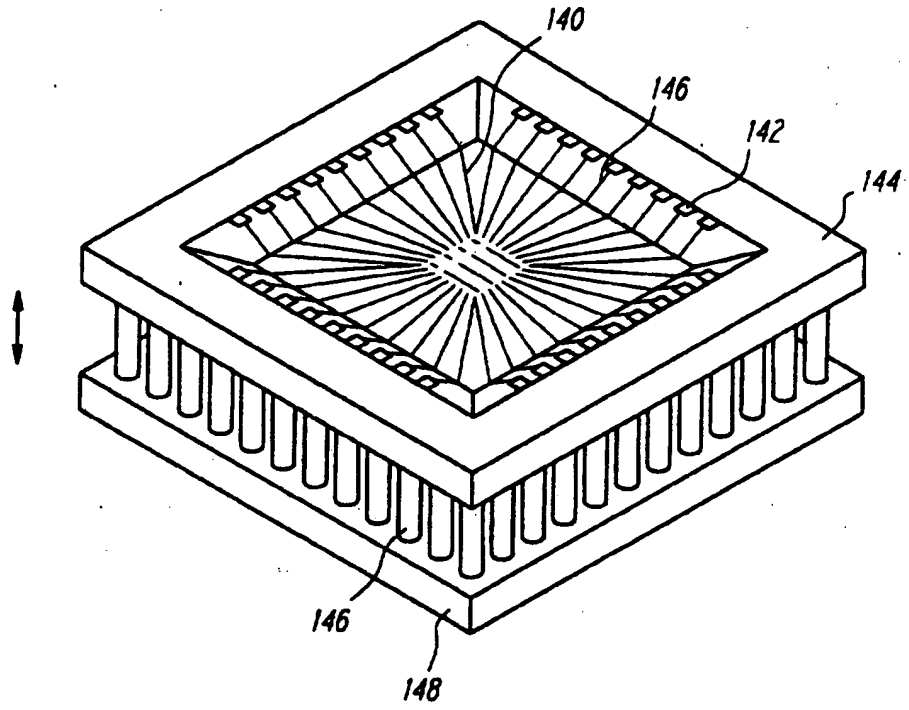


FIG. 10

FIG. 11A

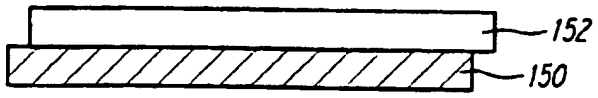


FIG. 11B

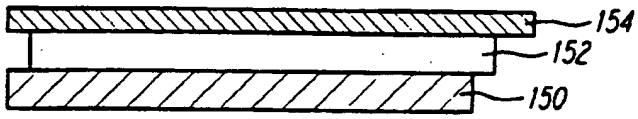


FIG. 11C

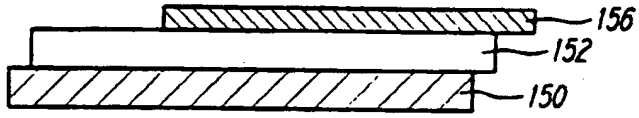


FIG. 11D

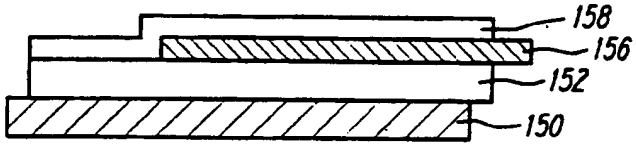


FIG. 11E

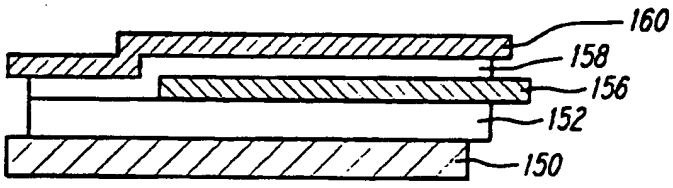


FIG. 11F

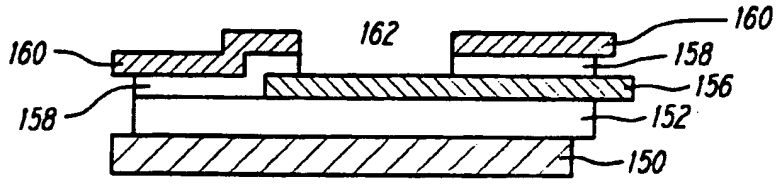
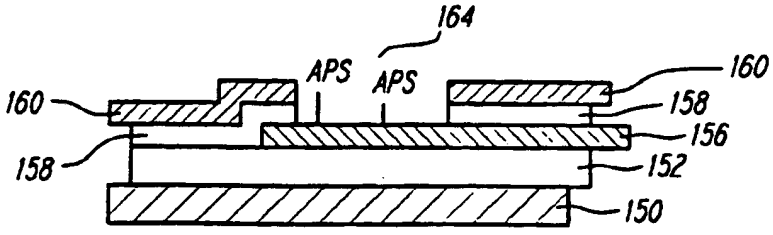


FIG. 11G



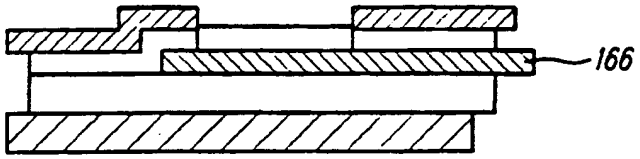


FIG. 12

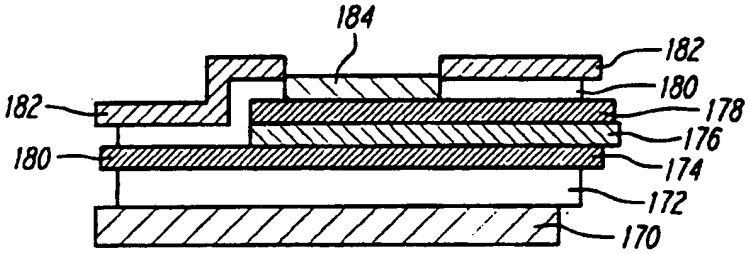


FIG. 13

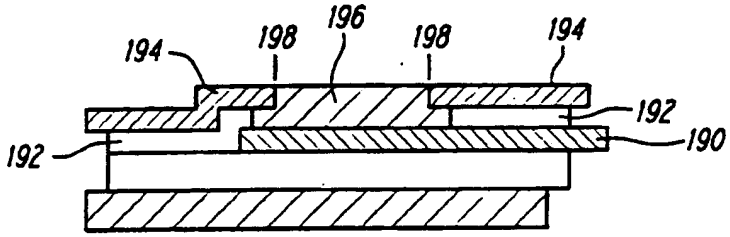


FIG. 14

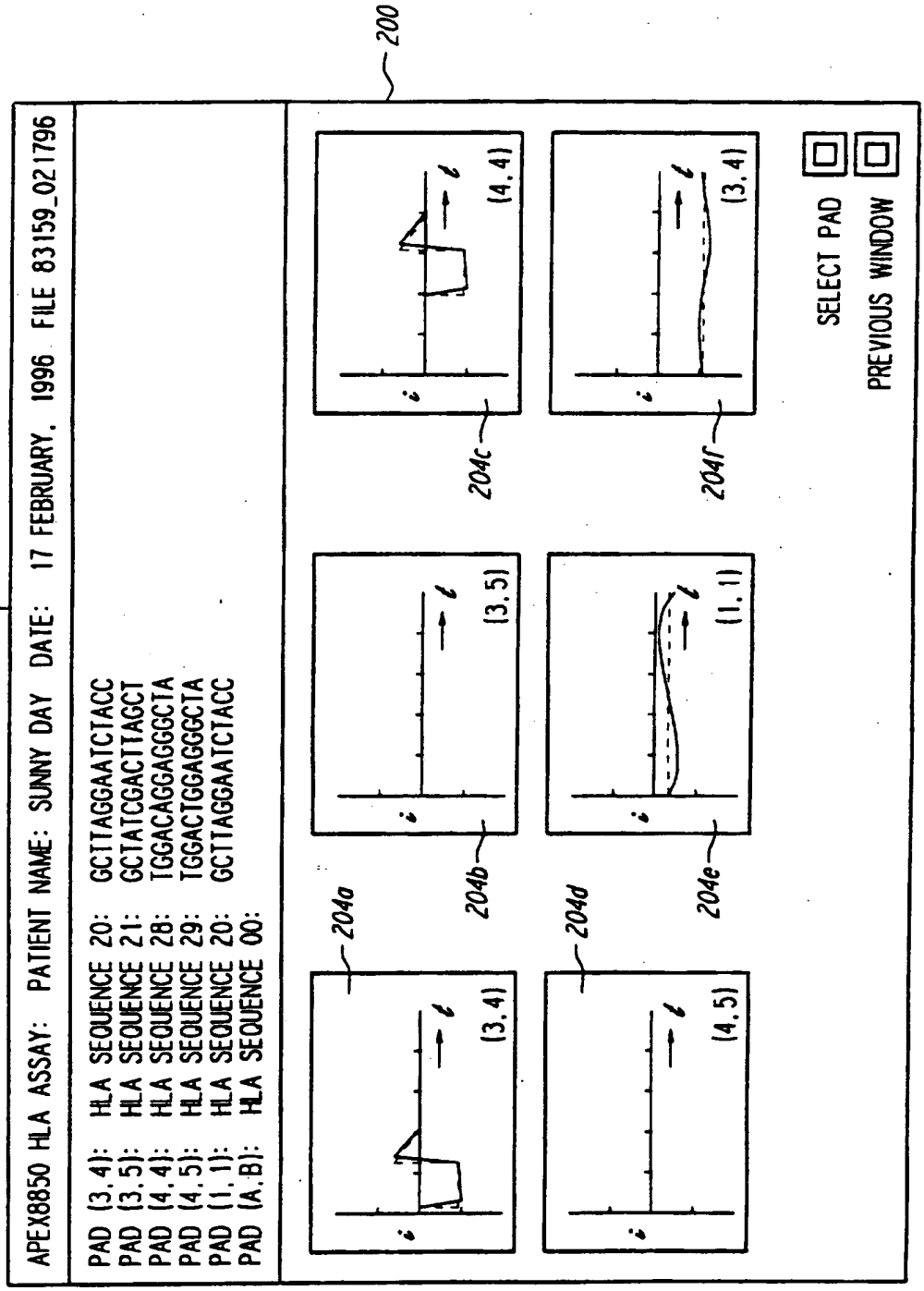

202
APEX8850 HLA ASSAY: PATIENT NAME: SUNNY DAY DATE: 17 FEBRUARY, 1996 FILE 83159_021796
PAD (3,4): HLA SEQUENCE 20: GCTTAGGAATCTACC
PAD (3,5): HLA SEQUENCE 21: GCTATCGACTTAGCT
PAD (4,4): HLA SEQUENCE 28: TGGACAGGAGGGCTA
PAD (4,5): HLA SEQUENCE 29: TGGACTGGAGGGCTA
PAD (1,1): HLA SEQUENCE 20: GCTTAGGAATCTACC
PAD (A,B): HLA SEQUENCE 00:
200
204a
(3,4)
204b
(3,5)
204c
(4,4)
204d
(4,5)
204e
(1,1)
204f
(3,4)
SELECT PAD
PREVIOUS WINDOW

FIG. 15

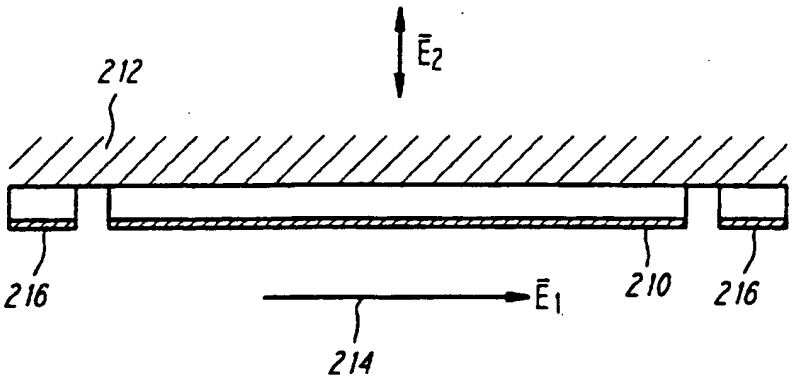


FIG. 16

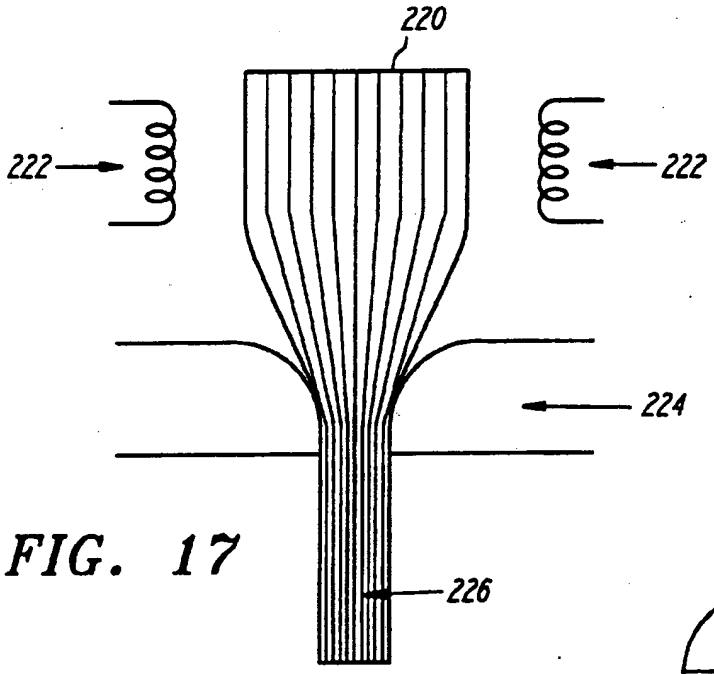


FIG. 17

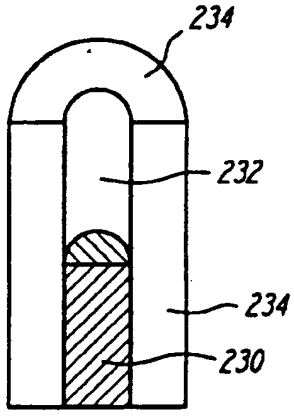


FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11333

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 35/00; C12P 19/34; C12M 1/00
US CL :422/50; 435/91.1,287
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/50,52,55,58,62,63,68.1,69,82.01,82.05,82.06,82.07,82.08,82.09,82.11,98; 435/6,7.1,91.1,91.2,287;536/22.1,25.3; 935/77,78,88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOSIS, MEDLINE,WPI, and BIOTECH ABS
search terms: device, microchip, biochip, reaction,PCR,assay,permeable,multiple,array

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US, A, 5,234,566 (OSMAN ET AL.) 10 AUGUST 1993, see especially the abstract, the Figures, Example 5, and claims 1-38.	1-10,13-31, 36-40,43, 49-54 ---------- 11,12,41, 42,44-48
X ---- Y	US, A, 5,118,605 (URDEA) 02 JUNE 1992, see especially the Figures and the DESCRIPTION OF THE SPECIFIC EMBODIMENTS in columns 3-15.	32-34 -------- 35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 DECEMBER 1995	Date of mailing of the international search report 03 JAN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARDIN MARSCHEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11333

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,200,051 (COZZETTE ET AL.) 06 APRIL 1993, see especially the abstract; Figures 4 and 8B and related discussion; column 18, lines 1-2; and column 26, lines 8-10.	1-10,13-31,36-40,43, 49-54 ——— 11,12,41, 42,44-48
Y	US, A, 5,096,669 (LAUKS ET AL.) 17 MARCH 1992, see the entire disclosure and especially claims 1-33.	9-13,17-31,36-54
X --- Y	US, A, 5,096,807 (LEABACK) 17 MARCH 1992, see especially the abstract, the Figures, and the discussion related to the Figures.	36,40,41, 43 ——— 1-31,37,42,44-54
X — Y	US, A, 5,227,265 (DEBOER ET AL.) 13 JULY 1993, see especially the abstract, the Figures, and the discussion related to the Figures.	36,40,41, 43 ——— 1-31,37,42,44-54
X, P —— Y, P	US, A, 5,304,487 (WILDING ET AL) 19 APRIL 1994, see especially the abstract, Figures, and claims 1-26.	32,36,40, 41,43- 49,51,52,54 ——— 1-31,33-35,37-39,42, 50,53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11333

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-17, drawn to electrodes with a contacting layer limitation.
Group II, claims 18-31, drawn to systems for controlling electrodes.
Group III, claims 32-35, drawn to DNA complexity reduction methods.
Group IV, claims 36-54, drawn to systems withan attachment layer but not limited to a contacting layer on electrodes.
The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The four groups lack a common special technical feature that links any two or more groups. Group I is directed to a device with a support, plurality of self-addressable electrodes with a contacting portion, and individual electrical connections. Such electrodes with a contacting portion are not a limiting feature of any of Groups II-IV. Group II is directed to a control system with a controller, input system, generator, and interface which may be utilized with a wide variety of devices wherein the devices arenot limited as given in the invention of Group I nor components of the system of Group IV. Group III is directed to DNA complexity reduction but is not limited to any device practice whatsoever and thus lacks any of the special technical device type features of Groups I, II, or IV. The electrophoretic force limitation of claim 35 in Group III is also not related to any device limitation of said Groups I, II, or IV. Lastly, Group IV has been noted above as lacking a special technical feature in common with each of Groups I-III. It is noted that the systems of Group IV are limited to containing devices with a plurality of separately addressable electrodes but that these electrodes are not described as self-addressable as in Group I and are thus not a common special technical feature as compared to the invention of Group I.

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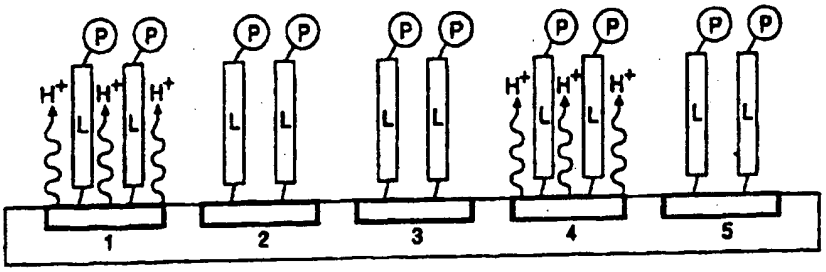

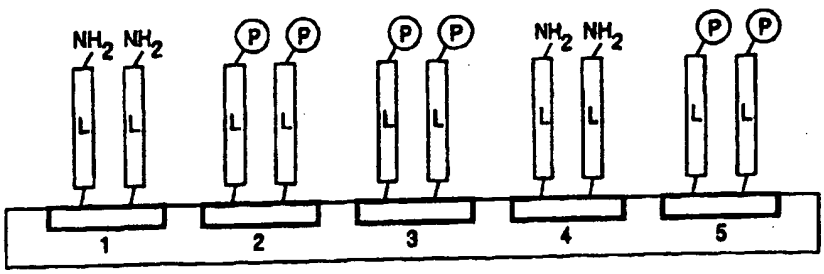

(57) Abstract

A solid synthesis method for the preparation of diverse sequences of separate polymers or nucleic acid sequences using electrochemical placement of monomers or nucleic acids at a specific location on a substrate containing at least one electrode (1-5) that is preferably in contact with a buffering or scavenging solution to prevent chemical crosstalk between electrodes (1-5) due to diffusion of electrochemically generated reagents.

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ELECTROCHEMICAL SOLID PHASE SYNTHESIS OF POLYMERS

Field of the Invention

The present invention is directed to the synthesis and placement of materials at select locations on a substrate. In particular, the present invention is directed to a method for providing separate sequences of chemical monomers at select locations on a substrate.

The present invention may be applied in the field of, but is not limited to, the preparation of peptide, oligomer, polymer, oligosaccharide, nucleic acid, ribonucleic acid, porphyrin, and drug congeners. In particular, the present invention may be used as a method to create sources of chemical diversity for use in screening for biological activity, for example, for use in the rapidly developing field of combinatorial chemistry.

Background of the Invention

There are many known assays for measuring the binding capabilities of known target molecules and the various molecules known to bind selectively to target molecules, i.e., ligands. The information that may be gained from such experiments often is limited by the number and type of ligands that are available. Continuing research is focused on the discovery of new ligands. Novel ligands are sometimes discovered by chance, or by application of techniques for the elucidation of molecular structure, or by systematic analysis of the relationships between molecular structure and binding activity.

Small peptide molecules are useful model systems for exploring the relationship between structure and function in biology. A peptide is a sequence of amino acids. For example, the twenty naturally occurring amino acids can be condensed into polymeric molecules. These polymeric molecules form a large variety of three-dimensional spatial and electronic structures. Each structure arises from a particular amino acid sequence and solvent condition. The number of possible hexapeptides of the twenty naturally

occurring amino acids, for example, is 20^6, or 64 million different peptides. As shown by epitope studies, the small peptide molecules are useful in target-binding studies, and sequences as short as a few amino acids are recognized with high specificity by some antibodies.

The process of discovering ligands with desirable patterns of specificity for targets of biological importance is central to many contemporary approaches to drug discovery. These approaches, based on structure-activity relationships, involve rational design of ligands and large scale screening of families of potential ligands. Often, a combination of approaches is used. The ligands are often, but not exclusively, small peptide molecules.

Yet methods of preparing large numbers of different ligands have been painstakingly slow and prohibitively expensive when used at a scale sufficient to permit effective rational or random screening. For example, the well-known "Merrifield" method (*J. Am. Chem. Soc.* (1963) 85:2149-2154), which is incorporated herein by reference, has been used to synthesize peptides on solid supports. In the Merrifield method, an amino acid is bound covalently to a support made of an insoluble polymer. Another amino acid with an alpha protected group is reacted with the covalently bonded amino acid to form a dipeptide. After washing, the protective group is removed and a third amino acid with an alpha protective group is added to the dipeptide. This process is continued until a peptide of a desired length and sequence is obtained. Using the Merrifield method, synthesis of more than a handful of peptide sequences in a day is not technically feasible or economically practical.

To synthesize larger numbers of polymer sequences, it has been proposed to use a series of reaction vessels for polymer synthesis. For example, a tubular reactor system may be used to synthesize a linear polymer on a solid phase support by automated sequential addition of reagents. This method, however, also does not enable the synthesis of a sufficiently large number of polymer sequences for effective and economical screening.

Another method of preparing a plurality of polymer sequences uses a porous container enclosing a known quantity of reactive particles, larger in size

than pores of the container. The particles in the containers may be selectively reacted with desired materials to synthesize desired sequences of product molecules. However, as with the other methods known in the art, this method is not practical for the synthesis of a sufficient variety of polypeptides for effective screening.

Other techniques have also been described and attempted. Several of these methods include synthesis of peptides on 96 plastic pins that fit the format of standard microtiter plates. Unfortunately, while these techniques have been somewhat useful, substantial problems remain. For example, methods using standard microtiter plates continue to be limited in the diversity of sequences that can be synthesized and screened. Although it is recognized that use of microtiter plates produces essentially pure polymers because each polymer is synthesized in an isolated well of the microtiter plate, the number of polymers that can be produced in any given time is limited by the number of wells in a microtiter plate, i.e., 96. Moreover, the equipment needed for synthesis in the microtiter plates is large. Because of this limitation, use of microtiter plates requires a large amount of space to produce a relatively small number of peptides.

One attempt at synthesizing a large number of diverse arrays of polypeptides and polymers in a smaller space is found in U.S. Patent No. 5,143,854 granted to Pirrung et al. (1992). This patent describes the use of photolithographic techniques for the solid phase synthesis of arrays of polypeptides and polymers. The disclosed technique uses "photomasks" and photolabile protecting groups for protecting the underlying functional group. Each step of the process requires the use of a different photomask to control which regions are exposed to light and thus deprotected. The necessity of having to fabricate a new set of photomasks for each array of chemical monomers results in a method that is extremely expensive and not well-suited to automation. Moreover, this method is tedious and time consuming because each step of the synthesis requires the mechanical removal, replacement and realignment of a photomask. Thus, synthesizing a large number of libraries of

polymers with the Pirrung method is an inefficient and uneconomical process.

Another drawback of the Pirrung method is that the photolabile protecting groups used cannot be removed as effectively as conventional acid or base labile protecting groups can be removed and are plagued by contamination due to undesired side reactions. Consequently, using Pirrung's method, the purity of the chemical array is often compromised due to incomplete removal of the protecting groups and subsequent failure of the underlying functional groups to react with the desired monomer, as well as contamination from undesired side reactions.

Another attempt to synthesize large numbers of polymers is disclosed by Southern in International patent application WO 93/22480, published November 11, 1993. Southern describes a method for synthesizing polymers at selected sites by electrochemically modifying a surface; this method involves providing an electrolyte overlaying the surface and an array of electrodes adjacent to the surface. In each step of Southern's synthesis process, an array of electrodes is mechanically placed adjacent the points of synthesis, and a voltage is applied that is sufficient to produce electrochemical reagents at the electrode. The electrochemical reagents are deposited on the surface themselves or are allowed to react with another species, found either in the electrolyte or on the surface, in order to deposit or modify a substance at the desired points of synthesis. The array of electrodes is then mechanically removed and the surface is subsequently contacted with selected monomers. For subsequent reactions, the array of electrodes is again mechanically placed adjacent the surface and a subsequent set of selected electrodes activated.

This method requires that a large amount of control be exercised over the distance that exists between the electrode array and the surface where synthesis occurs. Control over the distance between the electrodes and the surface for modification is required to ensure precise alignment between the electrodes and the points of synthesis and to limit the extent of diffusion of electrochemically generated reagents away from the desired points of synthesis. However, the inherent difficulty in positioning electrodes repeatedly

and accurately within a few microns of the surface frequently results in the production of electrochemically generated reagents at undesirable synthesis points. Moreover, the diffusion of the electrochemically generated reagents from desired sites of reaction to undesired sites of reaction results in "chemical cross-talk" between synthesis sites. This cross-talk severely compromises the purity of the final product, as undesired binding reactions occur at unselected sites. The amount of cross-talk is further aggravated by the disruptions of surface tension that occur whenever the electrodes are moved, leading to convective mixing that causes increased movement of the electrochemically generated reagents. While Southern attempts to minimize the amount of cross-talk by applying a potential designed to counteract diffusion, as a practical matter, the electric fields Southern can generate are too low to prevent diffusion. When the potential is raised to increase the electric field, large quantities of undesired electrochemically generated reagents are produced. Hence, Southern is not a practical method for generating large numbers of pure polymers.

A more recent attempt to automate the synthesis of polymers is disclosed by Heller in International patent application WO 95/12808, published May 11, 1995. Heller describes a self-addressable, self-assembling microelectronic system that can carry out controlled multi-step reactions in microscopic environments, including biopolymer synthesis of oligonucleotides and peptides. The Heller method employs free field electrophoresis to transport analytes or reactants to selected micro-locations where they are effectively concentrated and reacted with the specific binding entities. Each micro-location of the Heller device has a derivatized surface for the covalent attachment of specific binding entities, which includes an attachment layer, a permeation layer, and an underlying direct current micro-electrode. The presence of the permeation layer prevents any electrochemically generated reagents from interacting with or binding to either the points of synthesis or to reagents that are electrophoretically transported to each synthesis site. Thus, all synthesis is due to reagents that are electrophoretically transported to each

site of synthesis.

The Heller method, however, is severely limited by the use of electrophoretic transport. First, electrophoretic transport requires that the reactants be charged in order to be affected by the electric fields; however, conventional reactants of interest for combinatorial chemistry are usually uncharged molecules not useable in an electrophoretic system. Second, the Heller method does not, and cannot, address the large amount of chemical crosstalk that inherently occurs because of the spatial distribution of the electric fields involved in the electrophoretic transport of the reagents for binding. In a system utilizing electrophoresis, one cannot use protecting groups to protect the reactive functional groups at the microlocations since there is no mechanism for removing the protective groups; yet, the use of electrophoresis results in various binding entities and/or reactants being located throughout the solution used as they migrate, often coming into contact with unselected reaction sites. Thus, the combination of the lack of protecting groups and the spatial distribution of the electric fields inherent to electrophoresis allow such binding reactions to occur randomly, compromising the fidelity of any polymer being synthesized.

From the above, it is seen that there is an existing need and desire for an improved method for synthesizing a variety of chemical sequences at known locations that uses highly efficient deprotection and coupling mechanisms. It is further seen that there is an existing need and desire for a method for synthesizing a variety of chemical sequences at known locations that is cost-effective and practical, and which allows use of a smaller sized apparatus affording more efficient production in a specific area and time, while maintaining the fidelity of the chemical sequences produced. As should be clear to those skilled in the art, the above discussion directed to polypeptide synthesis from monomers is equally applicable to oligonucleotide, and more specifically, deoxyribonucleic acid (DNA) synthesis from deoxyribonucleotide monomers.

It is therefore an object of the present invention to provide an improved method for the placement of a material at a specific location on a substrate. It is further an object of the present invention to provide an improved method for the rapid synthesis of an array of separate, diverse and pure polymers or oligonucleotides on a substrate.

It is still a further object of the invention to provide a substrate for separate and pure polymer or oligonucleotide or DNA synthesis that contains a multi-electrode array that allows electrodes to be placed in very close proximity for use in combinatorial chemistry. It is still another object of the invention to provide a substrate for separate and pure polymer or DNA synthesis that contains a multi-electrode array of electrodes in very close proximity, that allows for automation of a polymer or DNA synthesis process, and which can be used in functional genomics, diagnostics, gene screening, drug discovery and screening for materials useful for research, industrial, commercial and therapeutic uses.

Additional features and advantages of the present invention will be set forth in part in the description that follows, and in part will be apparent from the description, or may be learned by practice of the invention. The objectives and other advantages of the present invention will be realized and attained by means of the elements and combinations particularly pointed out in the written description and appended claims.

Summary of the Invention

The foregoing objects have been accomplished in accordance with this invention by providing a method for electrochemical placement of a material at a specific location on a substrate, which comprises the steps of:

providing a substrate having at its surface at least one electrode that is proximate to at least one molecule bearing at least one protected chemical functional group,

applying a potential to the electrode sufficient to generate electrochemical reagents capable of deprotecting at least one of the protected

chemical functional groups of the molecule, and

bonding the deprotected chemical functional group with a monomer or a pre-formed molecule.

The present invention also includes a method for electrochemical synthesis of an array of separately formed polymers on a substrate, which comprises the steps of:

placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more molecules bearing at least one protected chemical functional group attached thereto,

selectively deprotecting at least one protected chemical functional group on at least one of the molecules;

bonding a first monomer having at least one protected chemical functional group to one or more deprotected chemical functional groups of the molecule;

selectively deprotecting a chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group;

bonding a second monomer having at least one protected chemical functional group to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule; and

repeating the selective deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule and the subsequent bonding of an additional monomer to the deprotected chemical functional group until at least two separate polymers of desired length are formed on the substrate surface.

Another embodiment of the present invention also includes a method for electrochemical synthesis of an array of separately formed oligonucleotides on a substrate, which comprises the steps of:

placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being

proximate to one or more molecules bearing at least one protected chemical functional group attached thereto,

selectively deprotecting at least one protected chemical functional group on at least one of the molecules;

bonding a first nucleotide having at least one protected chemical functional group to one or more deprotected chemical functional groups of the molecule;

selectively deprotecting a chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group;

bonding a second nucleotide having at least one protected chemical functional group to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule; and

repeating the selective deprotection of a chemical functional group on a bonded protected nucleotide or a bonded protected molecule and the subsequent bonding of an additional nucleotide to the deprotected chemical functional group until at least two separate oligonucleotides of desired length are formed on the substrate surface.

By using the electrochemical techniques discussed herein, it is possible to place monomers, both those that can be used for polymer synthesis and those that can be decorated, and pre-formed molecules at small and precisely known locations on a substrate. It is therefore possible to synthesize polymers of a known chemical sequence at selected locations on a substrate. For example, in accordance with the presently disclosed invention, one can place nucleotides at selected locations on a substrate to synthesize desired sequences of nucleotides in the form of, for example, oligonucleotides.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the present invention, as claimed.

Brief Description of the Drawings

FIGURES 1a and 1b illustrate selective deprotection by electrochemically generated reagents (protons) generated at electrodes 1 and 4 to expose reactive functionalities (NH_2) on linker molecules (L) proximate electrodes 1 and 4. The substrate is shown in cross section and contains 5 electrodes.

FIGURES 2a and 2b illustrate the bonding of monomers (A) bearing protected chemical functional groups (P) with the deprotected linker molecules (bearing reactive functionalities) proximate electrodes 1 and 4.

FIGURES 3a and 3b illustrate selective deprotection by protons generated at electrodes 2 and 4 of a second set of reactive functionalities on the molecule and monomer proximate electrodes 2 and 4, respectively.

FIGURES 4a and 4b illustrate the bonding of monomers (B) bearing protected chemical functional groups (P) with the deprotected molecule and monomer proximate electrodes 2 and 4, respectively.

FIGURE 5 illustrates a 5 electrode substrate bearing all possible combinations of monomers (A) and (B). The linker molecule proximate electrode 1 has a protected dimer, e.g., a dipeptide, containing two (A) monomers bonded thereto. The linker molecule proximate electrode 2 has a protected dimer containing a (B) monomer bonded to the linker molecule (L) and a protected (A) monomer bonded to said (B) monomer. The linker molecule proximate electrode 3, which represents a control electrode, demonstrates a linker molecule where no synthesis occurs because no potential is applied to the proximate electrode. The linker molecule proximate electrode 4 has a protected dimer containing an (A) monomer bonded to a linker molecule (L) and a protected (B) monomer bonded to said (A) monomer. The linker molecule proximate electrode 5 has a protected dimer containing two (B) monomers bonded to a linker molecule (L).

FIGURE 6 illustrates a top view diagram of a substrate having at its surface a 10x10 electrode array, having 100 electrodes. A side view of an exemplary electrode at the surface of the substrate is also shown.

FIGURE 7 illustrates a substrate having a permeable attachment layer or membrane having CBZ-protected leucine monomers (L) bonded thereto. The layer/membrane overlays the electrodes at the surface of the substrate.

FIGURE 8 illustrates a substrate having a permeable attachment layer or membrane overlaying the electrodes at the surface, which layer/membrane contains leucine monomers (L) bearing reactive amine functionalities, e.g., following removal of protecting groups (P=CBZ) at monomers proximate electrodes 2, 3, 5, 6, and 7 and counter electrodes 1 and 10.

FIGURE 9 illustrates modification of monomers proximate electrodes 2, 3, 5, 6, and 7 following CBZ-protected phenylalanine monomers (F) have bonded with the reactive amine functionalities on the leucine monomers proximate these electrodes (a dipeptide is formed).

FIGURE 10 illustrates modification of the substrate surface by CBZ-protected tripeptides, glycine-phenylalanine-leucine (G-F-L) proximate electrodes 3, 5, 6, and 7.

FIGURE 11 illustrates modification of the substrate surface by CBZ-protected pentapeptides, tyrosine-glycine-glycine-phenylalanine-leucine (Y-G-G-F-L) proximate electrodes 6 and 7.

FIGURE 12 illustrates a protected leu-enkephalin epitope proximate electrode 7 and counter electrodes 1 and 10, and a deprotected leu-enkephalin epitope proximate electrode 6.

FIGURE 13 illustrates representative results as would be observed using an epifluorescent microscope following exposure to the antibody and fluorescent conjugate in accordance with Example 1.

FIGURE 14 is a digitally captured white light photomicrograph of an uncoated electrode array chip showing approximately seventy electrodes. This photomicrograph was taken using a 4x objective by an Olympus BX60 microscope with a Pulnix TM-745 integrating CCD camera. Note, there is electrical circuitry associated with these independently addressable electrodes.

FIGURE 15 is a digitally captured epifluorescent photomicrograph of the same array of electrodes pictured in FIGURE 14, at the same magnification.

This photomicrograph shows that on an uncoated electrode array chip, without any fluorescent coating material thereon, the electrodes are dark. The darkness of the electrodes is explained by the metal of the electrode (platinum) quenching any fluorescence present.

FIGURE 16 is a digitally captured epifluorescent photomicrograph of electrodes in the same array as in FIGURES 14 and 15, but taken using a 10x objective and showing only sixteen electrodes. This photomicrograph is of a chip that is coated with a fluorescent membrane material, i.e., there are fluorescent labeled molecules attached to a membrane overlaying the electrodes. This photomicrograph shows that when the electrodes are coated with a membrane containing florescent material, the area proximate/over the electrodes is bright. The fluorescent material used for this photomicrograph was streptavidin molecules labeled with Texas Red dye.

FIGURE 17 is a digitally captured white light photomicrograph similar to FIGURE 14, except that these electrodes are hard wired, as shown by the leads connecting the electrodes to the electrical source located off the micrograph. In addition, this photomicrograph was taken using a 10x objective. These hardwired electrodes are located on the side of the electrode array chips. Note, there is no circuitry associated with these hard wired-electrodes.

FIGURES 18a and 18b depict the chip/pin grid array (PGA) package assembly. As is shown in FIGURE 18a, the chip is attached to the PGA package with glue on the opposite side of the chip from the active area (active area is the area having electrodes at its surface), which leaves the active electrode area protruding from the end of the PGA package in a manner that allows the active area of the chip to be dipped or immersed into solutions. The electrical wires that connect the bond pads on the chip to the bond pads on the PGA package are encased in epoxy. The pins shown in FIGURE 18b are located on the opposite side of the PGA package shown in FIGURE 18a.

FIGURES 19a and 19b represent digitally captured epifluorescent photomicrographs showing an electrode array chip before (FIGURE 19a) and after (FIGURE 19b) application of voltage and performance of a deprotection step. Prior to application of any voltage, a 0.05M aqueous sodium phosphate

buffer at a pH of 8.0 was placed in contact with all the electrodes of the array to enable production of electrochemical reagents. FIGURE 19b shows the electrode array after all of the electrodes in the array were exposed to the same voltage and deprotection occurred at each electrode in the array. A voltage of 2.8 volts was applied for 10 minutes. This photomicrograph was taken using a 4x objective and using a 1 second integration time.

FIGURE 20 represents a digitally captured epifluorescent photomicrograph showing a hardwired electrode array chip wherein the anodes (the dark electrodes) and the cathodes were alternating electrodes. The depicted checkerboard pattern was obtained following application of 2.8 volts for 10 minutes. The objective used to obtain this photomicrograph was 4x and the integration time was 1 seconds. Note, the localization of the acid at the anodes. The precision of the localization achieved in accordance with the present invention allowed the checkerboard pattern to be obtained.

FIGURE 21 represents a digitally captured epifluorescent photomicrograph showing the same hardwired electrode array chip as in FIGURE 20, but this photomicrograph was taken using a 10x objective with a 700 millisecond integration time.

FIGURE 22 is a digitally captured epifluorescent photomicrograph of an uncoated electrode array chip showing an array of hardwired electrodes. (The neighboring electrode array is also shown in this figure.) The orientation of the array shown allows accurate reading of the brightness of the electrodes. The electrodes shown are dark. The three electrodes to which electrical connection was provided, and of which brightness or darkness observations were made, are labeled "T1", "T2", and "T4".

FIGURE 23 is a digitally captured epifluorescent photomicrograph of a chip that is coated with a fluorescent membrane containing Texas Red labeled streptavidin molecules that are attached to the electrodes via trityl linker molecules. Electrodes T2 and T4 have a strong bright signal. Electrode T1 is dark. No voltage has been applied to the electrodes yet.

FIGURE 24 is a digitally captured epifluorescent photomicrograph of the chip shown in FIGURE 23 after positive voltage has been applied to electrodes

T2 and T4. Positive voltage produced protons at these electrodes. Electrodes T2 and T4 are dark because the trityl linker molecule has dissociated from the membrane overlaying the electrodes. Electrode T1 was used as the counter electrode. Note that the dark areas are confined to electrodes T2 and T4, i.e., there is very little chemical cross talk occurring between neighboring electrodes.

FIGURES 25a and 25b represent digitally captured epifluorescent photomicrographs showing hardwired electrodes before (FIGURE 25a) and after (FIGURE 25b) a deprotection step performed in accordance with the reaction conditions, i.e., electrolyte, of the prior art, Southern WO 93/22480. These photomicrographs, taken through a 10x objective, show the imprecision and randomness caused by "chemical crosstalk" between the electrodes. The large areas of black-out and white-out surrounding the electrodes in these photomicrographs represent the excursion of the electrochemical reagents (protons) away from the electrode at which they were generated.

FIGURES 26a and 26b represent digitally captured epifluorescent photomicrographs taken through a 20x objective with a 100 millisecond integration time of the same hardwired electrodes as shown in FIGURES 25a and 25b.

Detailed Description of the Invention

The present invention provides methods for the preparation and use of a substrate having one or a plurality of chemical species in selected regions. The present invention is described herein primarily with regard to the preparation of molecules containing sequences of amino acids, but could be readily applied to the preparation of other polymers , as well as to the preparation of sequences of nucleic acids . Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either alpha-, beta-, or omega-amino acids, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this

disclosure. In a preferred embodiment, the invention herein is used in the synthesis of peptides. In another preferred embodiment, the present invention is used for the synthesis of oligonucleotides and/or DNA.

The present invention is directed to placing molecules, selected generally from monomers, linker molecules and pre-formed molecules, including, in particular, nucleic acids, at a specific location on a substrate. The present invention is more particularly directed to the synthesis of polymers at a specific location on a substrate, and in particular polypeptides, by means of a solid phase polymerization technique, which generally involves the electrochemical removal of a protecting group from a molecule provided on a substrate that is proximate at least one electrode. The present invention is also particularly directed to the synthesis of oligonucleotides and/or DNA at selected locations on a substrate, by means of the disclosed solid phase polymerization technique.

Electrochemical reagents capable of electrochemically removing protecting groups from chemical functional groups on the molecule are generated at selected electrodes by applying a sufficient electrical potential to the selected electrodes. Removal of a protecting group, or "deprotection," in accordance with the invention, occurs at selected molecules when a chemical reagent generated by the electrode acts to deprotect or remove, for example, an acid or base labile protecting group from the selected molecules.

In one embodiment of the present invention, a terminal end of a monomer, nucleotide, or linker molecule (i.e., a molecule which "links," for example, a monomer or nucleotide to a substrate) is provided with at least one reactive functional group, which is protected with a protecting group removable by an electrochemically generated reagent. The protecting group(s) is exposed to reagents electrochemically generated at the electrode and removed from the monomer, nucleotide or linker molecule in a first selected region to expose a reactive functional group. The substrate is then contacted with a first monomer or pre-formed molecule, which bonds with the exposed functional group(s). This first monomer or pre-formed molecule may also bear

at least one protected chemical functional group removable by an electrochemically generated reagent.

The monomers or pre-formed molecules can then be deprotected in the same manner to yield a second set of reactive chemical functional groups. A second monomer or pre-formed molecule, which may also bear at least one protecting group removable by an electrochemically generated reagent, is subsequently brought into contact with the substrate to bond with the second set of exposed functional groups. Any unreacted functional groups can optionally be capped at any point during the synthesis process. The deprotection and bonding steps can be repeated sequentially at this site on the substrate until polymers or oligonucleotides of a desired sequence and length are obtained.

In another embodiment of the present invention, the substrate having one or more molecules bearing at least one protected chemical functional group bonded thereto is proximate an array of electrodes, which array is in contact with a buffering or scavenging solution. Following application of an electric potential to selected electrodes in the array sufficient to generate electrochemical reagents capable of deprotecting the protected chemical functional groups, molecules proximate the selected electrodes are deprotected to expose reactive functional groups, thereby preparing them for bonding. A monomer solution or a solution of pre-formed molecules, such as proteins, nucleic acids, polysaccharides, and porphyrins, is then contacted with the substrate surface and the monomers or pre-formed molecules bond with the deprotected chemical functional groups.

Another sufficient potential is subsequently applied to select electrodes in the array to deprotect at least one chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group. A second monomer or pre-formed molecule having at least one protected chemical functional group is subsequently bonded to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule. The selective deprotection and bonding steps can be

repeated sequentially until polymers or oligonucleotides of a desired sequence and length are obtained. The selective deprotection step is repeated by applying another potential sufficient to effect deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule. The subsequent bonding of an additional monomer or pre-formed molecule to the deprotected chemical functional group(s) until at least two separate polymers or oligonucleotides of desired length are formed on the substrate. **FIGURES 1-5** generically illustrate the above-discussed embodiments.

Preferred embodiments of the present invention use a buffering or scavenging solution in contact with each electrode, which is buffered towards the electrochemically generated reagents, in particular, towards protons and/or hydroxyl ions, and which actively prevents chemical cross-talk caused by diffusion of the electrochemically generated ions from one electrode to another electrode in an array. For example, when an electrode exposed to an aqueous or partially aqueous media is biased to a sufficiently positive (or negative) potential, protons (or hydroxyl ions) are produced as products of water hydrolysis. Protons, for example, are useful for removing electrochemical protecting groups from several molecules useful in combinatorial synthesis, for example, peptides, nucleic acids, and polysaccharides.

In order to produce separate and pure polymers, it is desirable to keep these protons (or hydroxyl ions) confined to the area immediately proximate the selected electrode(s) in order to minimize, and, if possible to eliminate, chemical cross-talk between nearby electrodes in an array. The spatial extent of excursion of electrochemically generated reagents can be actively controlled by the use of a buffering or scavenging solution that reacts with the reagents that move away from the selected electrodes, thus preventing these reagents from reacting at a nearby electrode.

The present invention advantageously minimizes, and preferably eliminates, chemical cross-talk between nearby areas of polymer or nucleic acid sequence synthesis on a substrate, thus enabling the synthesis of

separate arrays of pure polymers or nucleic acid sequences in a small specified area on a substrate using conventional electrochemically generated reagents and known electrochemical reactions. The ability of the inventive methods to place materials at specific locations on a substrate enables the inventive method to be used in several areas of synthesis in addition to polymer synthesis. Several examples of this synthesis include DNA and oligonucleotide synthesis, monomer decoration, which involves the addition of chemical moieties to a single monomer, and inorganic synthesis, which involves the addition of, for example, metals to porphyrins.

Other embodiments of the present invention contemplate an array of electrodes of small micron size, for example, ranging from 1 to 100 microns in diameter, and separated by many microns. However, it is also contemplated that electrodes separated by only submicron distances can be used, if desired. This arrangement affords a large quantity of separate and pure polymers or nucleic acid sequences to be synthesized simultaneously in a small area on a substrate in accordance with the inventive method. This capability renders the inventive method easily automated. The ability of the present invention to be automated easily while retaining the capability of producing separate and diverse arrays of pure polymers and nucleic acid sequences makes the present invention ideal for use in the rapidly developing areas of combinatorial chemistry and functional genomics.

Essentially, any conceivable substrate may be employed in accordance with the present invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat, but may take on a variety of alternative structure configurations. For example, the substrate may contain raised or depressed regions on which synthesis may take place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate

and the area for synthesis of each individual polymer or small molecule may be of any size and shape. Moreover, a substrate may comprise different materials at different regions.

Contemplated materials, which are preferably used as substrates and which are capable of holding and insulating electrically the electrodes, include: undoped semiconductors, such as silicon nitride, silicon oxide, silicon, diamond, chalcopyrites, wurtzites, sphalerites, halites, Group III-V compounds, and Group II-VI compounds; glass, such as, cobalt glass, pyrex glass, vycor glass, borosilicate glass and quartz; ceramics, such as, alumina, porcelain, zircon, corderite, titanates, metal oxides, clays, and zeolites; polymers, such as, paralyene, high density polyethylene, teflons, nylons, polycarbonates, polystyrenes, polyacylates, polycyanoacrylates, polyvinyl alcohols, polyimides, polyamides, polysiloxanes, polysilicones, polynitriles, polyvinyl chlorides, alkyd polymers, celluloses, expoxy polymers, melamines, urethanes, copolymers and mixtures of any of the above with other polymers, and mixtures of any of the above with glass or ceramics; and waxes, such as, apeizon. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure.

The substrate of the invention is proximate to at least one electrode, i.e., an electrically conducting region of the substrate that is substantially surrounded by an electrically insulating region. The electrode(s), by being "proximate" to the substrate, can be located at the substrate, i.e., embedded in or on the substrate, can be next to, below, or above the substrate, but need to be in close enough proximity to the substrate so that the reagents electrochemically generated at the electrode(s) can accomplish the desired deprotection of the chemical functional groups on the monomer(s) and/or molecule(s).

In addition to being proximate to at least one electrode, the substrate has on a surface thereof, at least one molecule, and preferably several molecules, bearing at least one chemical functional group protected by an electrochemically removable protecting group. These molecules bearing

protected chemical functional groups also need to be proximate to the electrode(s). In this regard, the molecules on the surface of the substrate need to be in close enough proximity to the electrode(s) so that the electrochemical reagents generated at the electrode can remove the protecting group from at least one protected functional group on the proximate molecule(s).

The molecules bearing a protected chemical functional group that are attached to the surface of the substrate may be selected generally from monomers, linker molecules and pre-formed molecules. Preferably, the molecules attached to the surface of the substrate include monomers, nucleotides, and linker molecules. All of these molecules generally bond to the substrate by covalent bonds or ionic interactions. Alternatively, all of these molecules can be bonded, also by covalent bonds or ionic interactions, to a layer overlaying the substrate, for example, a permeable membrane layer, which layer can be adhered to the substrate surface in several different ways, including covalent bonding, ionic interactions, dispersive interactions and hydrophilic or hydrophobic interactions. In still another manner of attachment, a monomer or pre-formed molecule may be bonded to a linker molecule that is bonded to either the substrate or a layer overlaying the substrate.

The monomers, linker molecules and pre-formed molecules used herein, are preferably provided with a chemical functional group that is protected by a protecting group removable by electrochemically generated reagents. If a chemical functional group capable of being deprotected by an electrochemically generated reagent is not present on the molecule on the substrate surface, bonding of subsequent monomers or pre-formed molecules cannot occur at this molecule. Preferably, the protecting group is on the distal or terminal end of the linker molecule, monomer, or pre-formed molecule, opposite the substrate. That is, the linker molecule preferably terminates in a chemical functional group, such as an amino or carboxy acid group, bearing an electrochemically removable protective group. Chemical functional groups that are found on the monomers, linker molecules and pre-formed molecules include any chemically reactive functionality. Usually, chemical functional

groups are associated with corresponding protective groups and will be chosen or utilized based on the product being synthesized. The molecules of the invention bond to deprotected chemical functional groups by covalent bonds or ionic interactions.

Monomers used in accordance with the present invention to synthesize the various polymers contemplated include all members of the set of small molecules that can be joined together to form a polymer. This set includes, but is not limited to, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomers include any member of a basis set for synthesis of a polymer. For example, trimers of L-amino acids form a basis set of approximately 8000 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer using the inventive method. The number of monomers that can be used in accordance with the inventive synthesis methods can vary widely, for example from 2 to several thousand monomers can be used, but in more preferred embodiments, the number of monomers will range from approximately 4 to approximately 200, and, more preferably, the number of monomers will range from 4-20.

Additional monomers that can be used in accordance with the invention also include the set of monomers that can be decorated, i.e., monomers to which chemical moieties can be added, such as prostaglandins, benzodiazapines, thromboxanes and leukotrienes. Combinations of monomers useful for polymer synthesis and monomers that can be decorated are also contemplated by the invention. The above-discussed monomers may be obtained in unprotected form from most any chemical supply company, and most, if not all, can be obtained in protected form from Bachem, Inc., Torrance, California. Phosphoramidite monomers for nucleic acid synthesis can be obtained from Applied Biosystems, Inc., Foster City, California.

In a preferred embodiment of the invention, the monomers are amino acids containing a protective group at its amino or carboxy terminus that is

removable by an electrochemically generated reagent. A polymer in which the monomers are alpha amino acids and are joined together through amide bonds is a peptide, also known as a polypeptide. In the context of the present invention, it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer or a mixture of the two. Peptides are at least two amino acid monomers long, and often are more than 20 amino acid monomers long.

Furthermore, essentially any pre-formed molecule can be bonded to the substrate, a layer overlaying the substrate, a monomer or a linker molecule. Pre-formed molecules include, for example, proteins, including in particular, receptors, enzymes, ion channels, and antibodies, nucleic acids, polysaccharides, porphyrins, and the like. Pre-formed molecules are, in general, formed at a site other than on the substrate of the invention. In a preferred embodiment, a pre-formed molecule is bonded to a deprotected functional group on a molecule, monomer, or another pre-formed molecule. In this regard, a pre-formed molecule that is already attached to the substrate may additionally bear at least one protected chemical functional group to which a monomer or other pre-formed molecule may bond, following deprotection of the chemical functional group.

Protective groups are materials that bind to a monomer, a linker molecule or a pre-formed molecule to protect a reactive functionality on the monomer, linker molecule or pre-formed molecule, which may be removed upon selective exposure to an activator, such as an electrochemically generated reagent. Protective groups that may be used in accordance with the present invention preferably include all acid and base labile protecting groups. For example, peptide amine groups are preferably protected by t-butyloxycarbonyl (BOC) or benzyloxycarbonyl (CBZ), both of which are acid labile, or by 9-fluorenylmethoxycarbonyl (FMOC), which is base labile. Additionally, hydroxy groups on phosphoramidites may be protected by dimethoxytrityl (DMT), which is acid labile. Exocyclic amine groups on nucleosides, in particular on phosphoramidites, are preferably protected by

dimethylformamidine on the adenosine and guanosine bases, and isobutyryl on the cytidine bases, both of which are base labile protecting groups. This protection strategy is known as fast oligonucleotide deprotection (FOD). Phosphoramidites protected in this manner are known as FOD phosphoramidites.

Additional protecting groups that may be used in accordance with the present invention include acid labile groups for protecting amino moieties: tert-butyloxycarbonyl, tert-amyloxycarbonyl, adamantyloxycarbonyl, 1-methylcyclobutyloxycarbonyl, 2-(p-biphenyl)propyl(2)oxycarbonyl, 2-(p-phenylazophenylyl)propyl(2)oxycarbonyl, α,α-dimethyl-3,5-dimethyloxybenzyloxy-carbonyl, 2-phenylpropyl(2)oxycarbonyl, 4-methyloxybenzyloxycarbonyl, benzyloxycarbonyl, furfuryloxycarbonyl, triphenylmethyl (trityl), p-toluenesulfenylaminocarbonyl, dimethylphosphinothioyl, diphenylphosphinothioyl, 2-benzoyl-1-methylvinyl, o-nitrophenylsulfenyl, and 1-naphthylidene; as base labile groups for protecting amino moieties: 9-fluorenylmethyloxycarbonyl, methylsulfonylethyloxycarbonyl, and 5-benzisoazolylmethyleneoxycarbonyl; as groups for protecting amino moieties that are labile when reduced: dithiasuccinoyl, p-toluene sulfonyl, and piperidino-oxycarbonyl; as groups for protecting amino moieties that are labile when oxidized: (ethylthio)carbonyl; as groups for protecting amino moieties that are labile to miscellaneous reagents, the appropriate agent is listed in parenthesis after the group: phthaloyl (hydrazine), trifluoroacetyl (piperidine), and chloroacetyl (2-aminothiophenol); acid labile groups for protecting carboxylic acids: tert-butyl ester; acid labile groups for protecting hydroxyl groups: dimethyltrityl; and basic labile groups for protecting phosphotriester groups: cyanoethyl.

As mentioned above, any unreacted deprotected chemical functional groups may be capped at any point during a synthesis reaction to avoid or prevent further bonding at such molecule. Capping groups "cap" deprotected functional groups by, for example, binding with the unreacted amino functions to form amides. Capping agents suitable for use in the present invention

include: acetic anhydride, n-acetylimidizole, isopropenyl formate, fluorescamine, 3-nitrophthalic anhydride and 3-sulfoproponic anhydride. Of these, acetic anhydride and n-acetylimidizole are preferred.

In accordance with the invention, the surface of the substrate is preferably provided with a layer of linker molecules. Linker molecules allow for indirect attachment of monomers or pre-formed molecules to the substrate or a layer overlaying the substrate. The linker molecules are preferably attached to an overlaying layer via silicon-carbon bonds, using, for example, controlled porosity glass (CPG) as the layer material. Linker molecules also facilitate target recognition of the synthesized polymers. Furthermore, the linker molecules are preferably chosen based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to approach more closely the synthesized polymer.

The linker molecules are preferably of sufficient length to permit polymers on a completed substrate to interact freely with binding entities exposed to the substrate. The linker molecules, when used, are preferably 6-50 atoms long to provide sufficient exposure of the functional groups to the binding entity. The linker molecules, which may be advantageously used in accordance with the invention include, for example, aryl acetylene, ethylene glycol oligomers containing from 2 to 10 monomer units, diamines, diacids, amino acids, and combinations thereof. Other linker molecules may be used in accordance with the different embodiments of the present invention and will be recognized by those skilled in the art in light of this disclosure.

According to another preferred embodiment, linker molecules may be provided with a cleavable group at an intermediate position, which group can be cleaved with an electrochemically generated reagent. This group is preferably cleaved with a reagent different from the reagent(s) used to remove the protective groups. This enables removal of the various synthesized polymers or nucleic acid sequences following completion of the synthesis by

way of electrochemically generated reagents. In particular, derivatives of the acid labile 4,4'-dimethyoxytrityl molecules with an exocyclic active ester can be used in accordance with the present invention. These linker molecules can be obtained from Perseptive Biosystems, Framingham, Massachusetts. More preferably, N-succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate is used as a cleavable linker molecule during DNA synthesis. The synthesis and use of this molecule is described in *A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules*, by Brian D. Gildea, James M. Coull and Hubert Koester, Tetrahedron Letters, Volume 31, No. 49, pgs 7095-7098 (1990). Alternatively, other manners of cleaving can be used over the entire array at the same time, such as chemical reagents, light or heat.

The use of cleavable linker groups affords dissociation or separation of synthesized molecules, e.g., polymers or nucleic acid sequences, from the electrode array at any desired time. This dissociation allows transfer of the, for example, synthesized polymer or nucleic acid sequence, to another electrode array or to a second substrate. The second substrate could contain bacteria and serve to assay the effectiveness of molecules made on the original electrode array at killing bacteria. Alternatively, the second substrate could be used to purify the materials made on the original electrode array. Obviously, those skilled in the art can contemplate several uses for transferring the molecules synthesized on the original electrode to a second substrate.

The molecules of the invention, i.e., the monomers, linker molecules and pre-formed molecules, can be attached directly to the substrate or can be attached to a layer or membrane of separating material that overlays the substrate. Materials that can form a layer or membrane overlaying the substrate, such that molecules can be bound there for modification by electrochemically generated reagents, include: controlled porosity glass (CPG); generic polymers, such as, teflons, nylons, polycarbonates, polystyrenes, polyacylates, polycyanoacrylates, polyvinyl alcohols, polyamides, polyimides, polysiloxanes, polysilicones, polynitriles, polyelectrolytes, hydrogels, epoxy polymers, melamines, urethanes and copolymers and mixtures of these and

other polymers; biologically derived polymers, such as, polysaccharides, polyhyaluric acids, celluloses, and chitons; ceramics, such as, alumina, metal oxides, clays, and zeolites; surfactants; thiols; self-assembled monolayers; porous carbon; and fullerine materials. The membrane can be coated onto the substrate by spin coating, dip coating or manual application, or any other art-acceptable form of coating.

Reagents that can be generated electrochemically at the electrodes fall into two broad classes: oxidants and reductants. There are also miscellaneous reagents that are useful in accordance with the invention. Oxidants that can be generated electrochemically include iodine, iodate, periodic acid, hydrogen peroxide, hypochlorite, metavanadate, bromate, dichromate, cerium (IV), and permanganate. Reductants which can be generated electrochemically include chromium (II), ferrocyanide, thiols, thiosulfate, titanium (III), arsenic (III) and iron (II). The miscellaneous reagents include bromine, chloride, protons and hydroxyl ions. Among the foregoing reagents, protons, hydroxyl ions, iodine, bromine, chlorine and the thiols are preferred.

In accordance with preferred embodiments of the present invention, a buffering and/or scavenging solution is in contact with each electrode. The buffering and/or scavenging solutions that may be used in accordance with the invention are preferably buffered toward, or scavenge, protons and/or hydroxyl ions, although other electrochemically generated reagents capable of being buffered and/or scavenged are clearly contemplated. The buffering solution functions to prevent chemical cross-talk due to diffusion of electrochemically generated reagents from one electrode in an array to another electrode in the array, while a scavenging solution functions to seek out and neutralize/deactivate the electrochemically generated reagents by binding or reacting with them. Thus, the spatial extent of excursion of electrochemically generated reagents can be actively controlled by the use of a buffering solution and/or a scavenging solution. In accordance with the invention, the buffering and scavenging solutions may be used independently or together. Preferably,

a buffering solution is used because the capacity of a buffering solution is more easily maintained, as compared with a scavenging solution.

Buffering solutions that can be used in accordance with the present invention include all electrolyte salts used in aqueous or partially aqueous preparations. Buffering solutions preferably used in accordance with the present invention include: acetate buffers, which typically buffer around pH 5; borate buffers, which typically buffer around pH 8; carbonate buffers, which typically buffer around pH 9; citrate buffers, which typically buffer around pH 6; glycine buffers, which typically buffer around pH 3; HEPES buffers, which typically buffer around pH 7; MOPS buffers, which typically buffer around pH 7; phosphate buffers, which typically buffer around pH 7; TRIS buffers, which typically buffer around pH 8; and 0.1 M KI in solution, which buffers the iodine concentration by the equilibrium reaction $I_2 + I^- \rightleftharpoons I_3^-$, the equilibrium coefficient for this reaction being around 10^{-2}.

Alternatively, or in combination with a buffering solution, a scavenging solution may be used that contains species such as ternary amines that function as hydroxyl ion scavengers or sulfonic acids that function as proton scavengers in nonaqueous media. The rate at which a reagent/species is scavenged depends both on the intrinsic rate of the reaction occurring and on the concentration of the scavenger. For example, solvents make good scavengers because they are frequently present in high concentrations. Most molecules scavenge in a nonselective way, however, some molecules, such as superoxide dismutase and horseradish peroxidase, scavenge in a selective manner.

Of particular interest to the present invention are scavenger molecules that can scavenge the different reactive species commonly generated, for example, by water hydrolysis at electrodes, including hydroxyl radicals, superoxides, oxygen radicals, and hydrogen peroxide. Hydroxyl radicals are among the most reactive molecules known; their rate of reaction is diffusion controlled, that is, they react with the first reactant/species they encounter. When hydroxyl radicals are generated by water hydrolysis, the first molecule

they usually encounter is a water molecule. For this reason, water is a rapid and effective scavenger of hydroxyl radicals. Superoxides are also a relatively reactive species, but can be stable in some nonaqueous or partially aqueous solvents. In aqueous media, superoxides rapidly react with most molecules, including water. In many solvents, they can be scavenged selectively with superoxidase dismutase.

Oxygen radicals are a family of oxygen species that exist as free radicals. They can be scavenged by a wide variety of molecules such as water or ascorbic acid. Hydrogen peroxide is a relatively mild reactive species that is useful, in particular, in combinatorial synthesis. Hydrogen peroxide is scavenged by water and many types of oxidizing and reducing agents. The rate at which hydrogen peroxide is scavenged depends on the redox potential of the scavenger molecules being used. Hydrogen peroxide can also be scavenged selectively by horseradish peroxidase. Another electrochemically generated species that can be scavenged is iodine. Iodine is a mild oxidizing reagent that is also useful for combinatorial synthesis. Iodine can be scavenged by reaction with hydroxyl ions to form iodide ions and hypoiodite. The rate at which iodine is scavenged is pH dependent; higher pH solutions scavenge iodine faster. All of the scavenger molecules discussed above may be used in accordance with the present invention. Other scavenger molecules will be readily apparent to those skilled in the art upon review of this disclosure.

In accordance with the present invention, the buffering solutions are preferably used in a concentration of at least 0.01 mM. More preferably, the buffering solution is present in a concentration ranging from 1 to 100mM, and still more preferably, the buffering solution is present in a concentration ranging from 10 to 100mM. Most preferably, the buffering solution concentration is approximately 30 mM. A buffering solution concentration of approximately 0.1 molar, will allow protons or hydroxyl ions to move approximately 100 angstroms before buffering the pH to the bulk values. Lower buffering solution concentrations, such as 0.00001 molar, will allow ion excursion of approximately several microns, which still may be acceptable distance

depending on the distance between electrodes in an array.

In accordance with the present invention, the concentration of scavenger molecules in a solution will depend on the specific scavenger molecules used since different scavenging molecules react at different rates. The more reactive the scavenger, the lower the concentration of scavenging solution needed, and vice versa. Those skilled in the art will be able to determine the appropriate concentration of scavenging solution depending upon the specific scavenger selected.

The at least one electrode proximate the substrate of the invention is preferably an array of electrodes. Arrays of electrodes of any dimension may be used, including arrays containing up to several million electrodes. Preferably, multiple electrodes in an array are simultaneously addressable and controllable by an electrical source. More preferably, each electrode is individually addressable and controllable by its own electrical source, thereby affording selective application of different potentials to select electrodes in the array. In this regard, the electrodes can be described as "switchable".

The arrays need not be in any specific shape, that is, the electrodes need not be in a square matrix shape. Contemplated electrode array geometries include: squares; rectangles; rectilinear and hexagonal grid arrays with any sort of polygon boundary; concentric circle grid geometries wherein the electrodes form concentric circles about a common center, and which may be bounded by an arbitrary polygon; and fractal grid array geometries having electrodes with the same or different diameters. Interlaced electrodes may also be used in accordance with the present invention. Preferably, however, the array of electrodes contains at least 100 electrodes in a 10x10 matrix. One embodiment of a substrate that may be used in accordance with the present invention having a 10x10 matrix of electrodes is shown in **FIGURE 6**. A side view of an electrode at the surface of the substrate is also shown.

More preferably, the array of electrodes contains at least 400 electrodes in, for example, an at least 20x20 matrix. Even more preferably, the array contains at least 2048 electrodes in, for example, an at least 64x32 matrix, and

still more preferably, the array contains at least 204,800 electrodes in, for example, an at least 640x320 array. Other sized arrays that may be used in accordance with the present invention will be readily apparent to those of skill in the art upon review of this disclosure.

Electrode arrays containing electrodes ranging in diameter from approximately less than 1 micron to approximately 100 microns (0.1 millimeters) are advantageously used in accordance with the present invention. Further, electrode arrays having a distance of approximately 10-1000 microns from center to center of the electrodes, regardless of the electrode diameter, are advantageously used in accordance with the present invention. More preferably, a distance of 50-100 microns exists between the centers of two neighboring electrodes.

As shown in the side view of **FIGURE 6**, the electrodes may be flush with the surface of the substrate. However, in accordance with a preferred embodiment of the present invention, the electrodes are hemisphere shaped, rather than flat disks. More specifically, the profile of the hemisphere shaped electrodes is represented by an arctangent function that looks like a hemisphere. Those skilled in the art will be familiar with electrodes of this shape. Hemisphere shaped electrodes help assure that the electric potential is constant across the radial profile of the electrode. That is, hemisphere shaped electrodes help assure that the electric potential is not larger near the edge of the electrode than in the middle of the electrode, thus assuring that the generation of electrochemical reagents occurs at the same rate at all parts of the electrode.

Electrodes that may be used in accordance with the invention may be composed of, but are not limited to, noble metals such as iridium and/or platinum, and other metals, such as, palladium, gold, silver, copper, mercury, nickel, zinc, titanium, tungsten, aluminum, as well as alloys of various metals, and other conducting materials, such as, carbon, including glassy carbon, reticulated vitreous carbon, basal plane graphite, edge plane graphite and graphite. Doped oxides such as indium tin oxide, and semiconductors such as

silicon oxide and gallium arsenide are also contemplated. Additionally, the electrodes may be composed of conducting polymers, metal doped polymers, conducting ceramics and conducting clays. Among the noble metals, platinum and palladium are especially preferred because of the advantageous properties associated with their ability to absorb hydrogen, i.e., their ability to be "preloaded" with hydrogen before being used in the methods of the invention.

The electrode(s) used in accordance with the invention may be connected to an electric source in any known manner. Preferred ways of connecting the electrodes to the electric source include CMOS switching circuitry, radio and microwave frequency addressable switches, light addressable switches, and direct connection from an electrode to a bond pad on the perimeter of a semiconductor chip.

CMOS switching circuitry involves the connection of each of the electrodes to a CMOS transistor switch. The switch is accessed by sending an electronic address signal down a common bus to SRAM (static random access memory) circuitry associated with each electrode. When the switch is "on", the electrode is connected to an electric source. This is a preferred mode of operation.

Radio and microwave frequency addressable switches involve the electrodes being switched by a RF or microwave signal. This allows the switches to be thrown both with and/or without using switching logic. The switches can be tuned to receive a particular frequency or modulation frequency and switch without switching logic. Alternatively, the switches can use both methods.

Light addressable switches are switched by light. In this method, the electrodes can also be switched with and without switching logic. The light signal can be spatially localized to afford switching without switching logic. This is accomplished, for example, by scanning a laser beam over the electrode array; the electrode being switched each time the laser illuminates it. Alternatively, the whole array can be flood illuminated and the light signal can

be temporally modulated to generate a coded signal. However, switching logic is required for flood illumination.

One can also perform a type of light addressable switching in an indirect way. In this method, the electrodes are formed from semiconductor materials. The semiconductor electrodes are then biased below their threshold voltage. At sufficiently low biases, there is no electrochemistry occurring because the electrons do not have enough energy to overcome the band gap. The electrodes that are "on" will already have been switched on by another method. When the electrodes are illuminated, the electrons will acquire enough energy from the light to overcome the band gap and cause electrochemistry to occur.

Thus, an array of electrodes can be poised to perform electrochemistry whenever they are illuminated. With this method, the whole array can be flood illuminated or each electrode can be illuminated separately. This technique is useful for very rapid pulsing of the electrochemistry without the need for fast switching electronics. Direct connection from an electrode to a bond pad on the perimeter of the semiconductor chip is another possibility, although this method of connection could limit the density of the array.

Electrochemical generation of the desired type of chemical species requires that the electric potential of each electrode have a certain minimum value. That is to say, a certain minimum potential is necessary, which may be achieved by specifying either the voltage or the current. Thus, there are two ways to achieve the necessary minimum potential at each electrode: either the voltage may be specified at the necessary value or the current can be determined such that it is sufficient to accommodate the necessary voltage. The necessary minimum potential value will be determined by the type of chemical reagent chosen to be generated. One skilled in the art can easily determine the necessary voltage and/or current to be used based on the chemical species desired. The maximum value of potential that can be used is also determined by the chemical species desired. If the maximum value of potential associated with the desired chemical species is exceeded, undesired chemical species may be resultantly produced.

The substrates prepared in accordance with the present invention will have a variety of uses including, for example, screening large numbers of polymers for biological activity. To screen for biological activity, for example, in the field of pharmaceutical drug discovery, the substrate is exposed to one or more receptors such as antibodies, whole cells, receptors on vesicles, lipids, or any one of a variety of other receptors. The receptors are preferably labeled with, for example, an electrochemical marker, an electrochemiluminescent marker, a chemiluminescent marker, a fluorescent marker, a radioactive marker, or a labeled antibody reactive with the receptor. The location of the marker on the substrate is detected with, for example, electrochemical, fluorescence or autoradiographic techniques. Through knowledge of the sequence of the material at the location where binding is detected, it is possible to determine quickly which sequence binds with the receptor and, therefore, the technique can be used to screen large numbers of peptides.

The present invention can also be used for therapeutic materials development, i.e., for drug development and for biomaterial studies, as well as for biomedical research, analytical chemistry and bioprocess monitoring. An exemplary application of the present invention includes diagnostics in which various ligands for particular receptors can be placed on a substrate and, for example, blood sera can be screened. Another exemplary application includes the placement of single or multiple pre-formed receptor molecules at selected sites on a substrate and, for example, drug screening could be conducted by exposing the substrate to drug candidate molecules to determine which molecules bind to which pre-formed receptor molecules.

Yet another application includes, for example, sequencing genomic DNA by the technique of sequencing by hybridization. Another contemplated application includes the synthesis and display of differing quantities of molecules or ligands at different spatial locations on an electrode array chip and the subsequent performance of dilution series experiments directly on the chip. Dilution series experiments afford differentiation between specific and non-specific binding of, for example, ligands and receptors. Non-biological

applications are also contemplated, and include the production of organic materials with varying levels of doping for use, for example, in semiconductor devices. Other examples of non-biological uses include anitcorrosives, antifoulants, and paints.

The present invention will further be clarified and illustrated by the following examples, which are intended to be purely exemplary of the invention.

EXAMPLES

EXAMPLE 1: Combinatorial Synthesis of the Leu-enkephalin epitope

Background

Endorphins are naturally occurring small peptides, e.g., including approximately 20-40 amino acids, that bind to opiate receptors in the brain. It has been discovered that most of the activity of endorphins is due to the last five amino acids on the peptides. These terminal pentapeptides are called enkephalins.

The immunofluorescent technique for detecting the leu-enkephalin epitope follows standard detection protocols. See for example. F. M. Ausubel et al., *Short Protocols in Molecular Biology*, Third edition, Unit 14, pgs. 14-23ff (1995). This assay requires a primary antibody, e.g., the 3-E7 monoclonal antibody, and a secondary antibody-fluorochrome conjugate specific to the source species of primary antibody, e.g., the goat anti-mouse fluorescent conjugate. The 3-E7 antibody is a mouse monoclonal antibody against β-endorphins that bind to leu-enkephalins. Both of the antibodies for this technique can be obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana.

For additional information regarding the 3-E7 monoclonal antibody, see, Meo, Tommaso, et al., "Monoclonal antibody to the message sequence Try-Gly-Gly-Phe of opioid peptides exhibits the specificity requirements of mammalian opioid receptors," *Proc. Natl. Acad. Sci USA* 80, pps. 4084-4088

(1983).

Preparation of an electrode array for use in combinatorial synthesis

An 10x10 platinum electrode array is used, as is shown in **FIGURE 6**. Columns 1 and 10 are used as counter electrodes. The active columns of the array are columns 2, 3, 5, 6 and 7. Columns 4, 8 and 9 are never activated in this synthesis.

The surface of the array is modified with a permeable membrane layer formed from controlled porosity glass (CPG) that is applied to the array by deposition of silicon dioxide under appropriate conditions in the semiconductor manufacturing process. The CPG forms a chemically inert membrane that is permeable to ions. This membrane is functionalized by silanation with chloromethyl silane. The chloromethyl silane groups are further modified by ethylene glycol linker molecules containing ten ethylene glycol moieties by reacting the silanized CPG membrane with a molecule containing ten ethylene glycol moieties and two amino groups at each end. This membrane provides a layer overlaying the surface of the array that is functionalized by amine groups that are, in turn, attached to the CPG matrix via a silane moiety. The diamino ethylene glycol molecules act as linker molecules (spacer groups) between the membrane and the epitope molecules which are formed.

Addition of protected functional groups to the membrane

The functionalized CPG membrane covered electrode array is exposed to a DMF solution of benzyloxycarbonyl (CBZ) protected l-leucine containing coupling reagents, such as, but not limited to, dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide, at room temperature for approximately two hours. This exposure produces a CPG membrane layer covering the array that is completely covered with CBZ-protected l-leucine moieties attached to the membrane layer by ethylene glycol linker molecules. This moiety covered membrane layer is shown in **FIGURE 7**. This is the bed of molecules on which the epitope molecule is built.

The moiety covered membrane layer is then washed three times with an aqueous 0.1 M phosphate buffer solution having a pH of 7.4.

Removal of the protecting groups (deprotection)

Removal of the CBZ protecting groups from the protected amino acids, i.e., deprotection, using electrochemically generated reagents (protons) is performed as follows.

Referring to the electrode array of **FIGURE 6**, a preconditioning step is performed: columns 2, 3, 5, 6, and 7 are biased negative with respect to columns 1 and 10, which serve as counter electrodes. There is no reference electrode in this system. The potential difference is approximately 3 volts, which voltage is applied for approximately 10 seconds. This preconditioning step causes hydroxyl ions to be formed at the electrodes with a negative bias and protons to be formed at the counter electrodes having a positive bias. This preconditioning step also causes protons to be reduced to hydrogen molecules at electrodes with a negative bias. The platinum electrodes absorb and hold some of these hydrogen molecules in the bulk metal.

Following the preconditioning step, the bias is then reversed. The electrodes of columns 1 and 10 (counter electrodes) are biased negative with respect to columns 2, 3, 5, 6, and 7. The potential difference is approximately 2.6 volts, which voltage is applied for approximately three seconds. This step causes protons to be formed at the electrodes with a positive bias both from hydrolysis of water and from oxidation of hydrogen molecules that are absorbed into the platinum electrodes during the preconditioning step. As a result of the preconditioning step and this subsequent step, the CBZ protecting groups are removed from the leucine amino acid moieties at the electrodes in columns 2, 3, 5, 6, and 7.

These two steps result in deprotected reactive amine moieties remaining attached to the leucine molecules at these sites (columns 2, 3, 5, 6, and 7) as illustrated in **FIGURE 8**.

Preparation of the membrane for coupling

To prepare the reactive amine moiety covered membrane for coupling CBZ-l-phenylalanine to the deprotected leucine groups, the following steps are performed:

The electrode array containing the reactive amine moiety covered membrane is washed twice with pure DMF. The electrode array is then exposed to a DMF solution containing CBZ-l-phenylalanine and coupling reagents, such as DCC at room temperature for approximately two hours. This step results in the electrodes of columns 2, 3, 5, 6, and 7 being modified with an CBZ-protected dipeptide of leucine and phenylalanine. This is shown in **FIGURE 9**.

The deprotection and coupling steps are then repeated at columns 3, 5, 6, and 7. That is, the electrode array is again exposed to an aqueous 0.1 M phosphate buffer solution having a pH of 7.4. The electrode array is then exposed to a DMF solution of CBZ- protected glycine and coupling reagents for approximately 2 hours at room temperature. This results in the electrodes in columns 3, 5, 6, and 7 being modified with the CBZ- protected tripeptide glycine-phenylalanine-leucine (G-F-L), as shown in **FIGURE 10**.

The deprotection and coupling steps are then repeated at columns 5, 6, and 7. That is, the electrode array is again exposed to an aqueous 0.1 M phosphate buffer solution having a pH of 7.4 and then exposed to a DMF solution of CBZ-protected glycine and coupling reagents for approximately two hours at room temperature. This results in the electrodes in columns 5, 6, and 7 being modified with the CBZ-protected tetrapeptide glycine-glycine-phenylalanine-leucine (G-G-F-L).

The deprotection and coupling steps are then repeated at columns 6 and 7 while the electrode array is again exposed to an aqueous 0.1 M phosphate buffer solution having a pH of 7.4. The electrode array is then exposed to a DMF solution of CBZ-protected-l-tyrosine and coupling reagents for approximately two hours at room temperature. This results in the electrodes in columns 6 and 7 being modified with the CBZ-protected pentapeptide tyrosine-glycine-glycine-phenylalanine-leucine (Y-G-G-F-L), as shown in **FIGURE 11**. This is the CBZ-protected version of the desired Leu-enkephalin epitope.

The deprotecting step is then repeated at columns 2, 3, 5, and 6, without a preconditioning step, to remove the CBZ protecting groups from the terminal amino acids of the combinatorial sequences. This procedure produces the following sequences:

Columns 1 and 10: modified with the protected Leu-enkephalin epitope (these are the counter electrodes).

Column 2: modified with the deprotected dipeptide F-L.

Column 3: modified with the deprotected tripeptide G-F-L.

Columns 4, 8 and 9: modified with the CBZ-protected leucine amino acid.

Column 5: modified with the deprotected tetrapeptide G-G-F-L.

Column 6: modified with the deprotected Leu-enkephalin epitope.

Column 7: modified with the CBZ-protected Leu-enkephalin epitope.

3-E7 monoclonal antibody assay

The modified electrode array, i.e., with the 10 modified columns, is exposed, via the Leu-enkephalin epitope detection technique discussed above, to the 3-E7 monoclonal antibody, followed by exposure to the goat anti-mouse fluorescent conjugate. The electrode array is then examined using an epifluorescent microscope. The expected results are shown in **FIGURES 12 and 13**. As is shown in **FIGURES 12 and 13**, the active Leu-enkephalin epitope is present proximate to the electrodes of column 6. (Column 6 is the only column modified with the deprotected Leu-enkephalin epitope.)

Note: The synthesis proceeds at the counter electrodes (electrodes 1 and 10) because protons are generated at the counter electrodes during each preconditioning (deprotecting) step. Since a preconditioning step is not performed in the final deprotection step, no protons are produced at the counter electrodes in the final step and a protected Leu-enkephalin epitope is produced at the counter electrodes which does not react upon exposure to the antibody and fluorescent conjugate.

EXAMPLE 2: Combinatorial Synthesis of Deoxyribonucleic acids

Background

The monomer units for combinatorial synthesis of DNA are called phosphoramidites. Phosphoramidites are linked together into a single strand nucleic acid polymer through phosphodiester bonds. Since the phosphorous is protected by a cyanoethyl ether moiety during synthesis, the bonds are phosphotriester bonds. The cyanoethyl group can be removed by a base at the end of synthesis to give the phosphodiester linkage. Phosphoramidites have two ends that are called 3' and 5' ends. The 3' end of one phosphoramidite will couple with the 5' end of another. Usually the 3' end is attached to a solid support and the 5' end is modified by another phosphoramidite to start the synthesis cycle. The 5' end is a hydroxy group that can be protected by a molecule called dimethyltrityl (DMT). DMT groups are acid labile protecting groups.

There are four naturally occurring deoxyribonucleotide monomers that form DNA polymers. They are adenosine (A), thymidine (T), cytosine (C), and guanosine (G). DNA is considered an acid because the phosphodiester groups that bind the monomers together are acidic. The nucleosides (A, T, C, G) are organic bases. DNA in nature is normally tens of millions to billions of base units long. A fifteen base unit long piece of DNA will be prepared in the following example. A piece of DNA of this length is known as a oligonucleotide. DNA molecules should be at least this long, otherwise it is very difficult to distinguish between them.

The nucleosides are protected because the exocyclic amine bases (A, C, G) are susceptible to depurination by acids. The protecting groups on these bases are base labile. There are three kinds of protecting groups on phosphoramidites. They are the DMT groups, which protect the 5' hydroxyl groups. the cyanoethyl ether groups, which protect the phosphorous, and the FOD (fast oligonucleotide deprotection) groups, which protect the exocyclic amines on the nucleoside bases. The DMT groups are acid labile and the others are base labile.

DNA is found in nature mostly as the "duplex" form having the famous double helix structure. This means that two single strands of DNA are bound together by interactions between the nucleoside bases. The nucleoside base T interacts with the nucleoside base A to form an A-T linkage. The nucleoside base C interacts with the nucleoside base G to form a C-G linkage. The A-T and the C-G interactions are the only stable interactions; other combinations are weak. Linkages that are not A-T or C-G can occur, and are called mismatches. When two complimentary single strands of DNA come together to form a duplex, this is called hybridization. When the single strands of DNA in a duplex come apart, the duplex DNA is said to have denatured. DNA duplexes typically denature when they are exposed to heat and/or low ionic strength aqueous solutions.

To determine whether or not a specific DNA sequence has been synthesized at a particular site, one uses probe strands of DNA that are complimentary with the strands that presumably were synthesized at that site. These probe strands are labeled covalently with a fluorescent dye. The probe strands will bind to DNA molecules on the surface with both the correct sequence and the incorrect sequence. However, the melting temperatures are much lower for the DNA duplexes that contain mismatches, i.e., non A-T and C-G links, than those that are complimentary, i.e., A-T and C-G links. Thus, upon heating, the probes forming duplexes with the incorrect DNA strands will denature first. By increasing the temperature to a level where all of the mismatched DNA duplexes have denatured, it is possible to detect only the DNA molecules with the correct sequence by observing the fluorescent dye using epifluorescent microscopy. Alternatively, the test surface can be washed with low ionic strength aqueous solutions. This has the same effect as raising the temperature and is more convenient experimentally.

Synthesis procedure

The electrode array is first modified with an acrylate/polyvinyl alcohol copolymer layer or membrane. The copolymer layer contains numerous pendant hydroxyl groups that are reactive toward phosphoramidites. The

polymer modified electrode array is then exposed to DMT- protected cytidine phosphoramidite and tetrazole at a concentration of 0.05 M in an anhydrous acetonitrile for 30 seconds at room temperature. The cytosine base and all of the other bases used in this example are protected using the FOD protecting scheme. (FOD protecting groups afford the best protection against depurination of exocyclic amines.) The array is then washed with anhydrous acetonitrile. Any unreacted hydroxyl groups on the surface are then capped by exposing the surface to an anhydrous acetonitrile solution of acetic anhydride and 1-methylimidizole for thirty seconds. This results in a surface modified everywhere with DMT protected C base units.

The trivalent phosphite linkage between the polymer and the phosphoramidite is oxidized to the more stable pentavalent phosphotriester linkage by electrochemically generated iodine. The iodine is produced electrochemically by the oxidation of iodide ions in an aqueous THF solution of potassium iodide. Iodine can be confined to the local area where it is formed by both an iodine buffering reaction and a scavenging reaction. Iodine is buffered by an equilibrium reaction with iodide ions to form the triiodide ion. The triiodide ion is not a useful reagent. Further, the solution can be buffered with respect to hydroxyl ions such that it is slightly basic. Iodine reacts with hydroxyl ions to form iodide ions and hypoiodite. Both of these chemical species are unreactive. Thus, hydroxyl ions serve as scavengers for iodine. Because the electrochemical oxidation of iodide ions to iodine can occur under conditions that also produce protons, the local environment can be made acidic while the iodine is being generated. There will be no scavenging in the acidic regions where iodine needs to be active. As a result, there are stable phosphotriester linkages to the polymer film only over those electrodes that electrochemically generate iodine. The unoxidized phosphite linked groups will eventually fall off after repeated exposure to the acetic anhydride capping solution.

The electrode array is next exposed to an aqueous 0.1 M sodium phosphate solution. A positive potential is applied for one second to first

selected areas and the DMT protecting groups are removed from the cytidine phosphoramidites in first selected areas. The array is then washed with anhydrous acetic anhydride. The reactive array is then exposed to a 0.05 M solution of thymidine phosphoramidite, T, and tetrazole in anhydrous acetonitrile for 30 seconds. The T nucleotides react with the C nucleotides at the first selected sites to form a C-T dimer. The remaining unreacted C nucleotides are capped and the phosphite linkages are reduced to phosphotriester linkages as outlined above.

This procedure is repeated at second, third, fourth, and so on, selected sites to synthesize combinatorially four different fifteenmer oligonucleotides at selected sites on the array. The array is then exposed to a 0.1 M aqueous ammonium hydroxide solution at 50°C for an hour. The FOD protecting groups and the cyanoethyl protecting groups on the phosphotriester are removed by the hydroxyl ions. The resulting array consists of single strands of the oligomer nucleic acids bound covalently to the polymer membrane.

Evaluation of the fidelity of the array

The fidelity of the combinatorial array is tested using four different fluorescently labeled oligonucleotide probes that are complimentary to the oligonucleotides synthesized on the array. The array is exposed to a first 100 nanomolar solution of a fluorescently labeled oligonucleotide probe in a 0.1 M sodium phosphate buffer at pH 7.2 at room temperature for thirty minutes. The array is then washed three times with a 0.1 M sodium phosphate buffer solution at pH 7.2. The array is then examined with an epifluorescent microscope. Bright spots appear in first areas where the oligonucleotide probe is present. To ensure that the oligonucleotide probe and its compliment actually hybridized, the array is washed several times with deionized water at 70°C for five minutes. Reexamination of the array with the epifluorescent microscope reveals a dark field. This means that the probe hybridized to its compliment and the results are not due to nonspecific absorption. The array is then exposed to a second 100 nanomolar solution of another fluorescently labeled oligonucleotide probe in 0.1 M aqueous sodium phosphate buffer at pH

7.2. The array is subsequently washed, examined with the epifluorescent microscope and then checked for nonspecific absorption. Bright spots appear in the second areas where the nucleotide probes are synthesized. The procedure is repeated for the third and fourth oligonucleotide sequences. The control areas will not bind the fluorescently labeled probe and become bright at any point in the assay.

EXAMPLE 3 and COMPARATIVE EXAMPLE 4

For the following example and comparative example, results were recorded and reproduced in the form of video photomicrographs that were captured digitally of the respective electrode array chips under various conditions.

Recording of Results - Taking of Pictures

The photomicrographs were taken using an Olympus BX60 microscope with a Pulnix TM-745 integrating CCD camera. The camera was controlled by, and the images were captured by, a Data Translation DT3155 video capture card run by a Pentium-based personal computer. The software that controlled the DT3155 card can easily be written by one of ordinary skill in the art.

Most of the photomicrographs were taken with a 10x objective that allowed approximately 16 electrodes to be seen in each image; however, for purposes of evaluation, the images were sometimes cropped to focus on the activity of the electrodes of interest. At times a 4x objective was also used. Two types of photomicrographs were taken. A few were taken using white light illumination. In these, the electrodes appear reflective. For example, see **FIGURE 14**. The majority of the photomicrographs were taken using epifluorescent illumination. In these, the electrodes appear dark in the photomicrographs when they are uncoated, i.e., when no fluorescent coating is present, because the metal of the electrodes, e.g., the platinum, quenches any fluorescence present.

Epifluorescent microscopy involves illuminating the electrode array chip from a position above the chip surface, along a path normal to the chip surface.

The illuminating beam is filtered to obtain a narrow band centered at the excitation wavelength of the fluorescent dye being used. The fluorescent dye used in the following example and comparative example was Texas Red, which has an absorption maximum at 595 nm. This dye emits a fluorescent light with an emission maximum at 615 nm when it is excited with light of approximately 595 nm. Texas Red can be obtained from Molecular Probes, Eugene Oregon. Filters in the Olympus BX60 microscope prevent the excitation light from traveling to the optical detector of the CCD camera. The Olympus BX60 microscope is equipped with an ancillary art-recognized instrumentation module to perform epifluorescent microscopy using Texas Red dye.

Exemplary photomicrographs taken using white illumination and epifluorescent illumination are shown in **FIGURES 14-16**. **FIGURES 14** and **15** depict an uncoated electrode array chip, while **FIGURE 16** depicts an electrode array chip coated with a fluorescent membrane.

Description and Preparation of the electrode array chips

The chips prepared and used in the following example and comparative example were rectangular devices with a 16 (in the x-direction) by 64 (in the y-direction) array of 100 micron diameter platinum electrodes. The total number of electrodes in these arrays was 1024. The dimensions of the chips were approximately 0.5 cm (x-direction) by 2.3 cm (y-direction), and the total surface area of the chips was approximately 1 square centimeter. The electrodes in each array were approximately 250 microns apart in the x-direction and approximately 350 microns apart in the y-direction, measured from the center of the electrodes.

Each electrode in the array was capable of being addressed independently using an SRAM cell (static random access memory), a standard art-recognized way to independently address electric circuitry in an array. The SRAM cell was located next to the electrodes in the electrical circuitry associated with electrode. Each electrode in the array had four separate switchable voltage lines that attached to it, allowing each electrode in the array to be switched independently from one voltage line to another. The voltage

was arbitrary and was set by an external voltage source.

In the chips used in the following example and comparative example, there were additionally 13 electrodes on the side of the chips that were hard wired to bond pads, meaning they were not switchable or independently addressable as were the electrodes in the 16x64 array. These 13 electrodes had no circuitry associated with them except for a single voltage line, and thus allowed protocols to be run on them without engaging the associated electrode array. These 13 electrodes were 100 microns in diameter and were spaced differently from the electrodes in the array. See, for example, **FIGURE 17**, showing the triangular orientation of the hard-wired electrodes, wherein the electrodes are 250 microns apart from the centers of the electrodes.

The chips were made by a 3 micron process using hybrid digital/analog very large scale integration (VLSI). One skilled in the art would be familiar with such a process and could easily prepare a chip for use in accordance with the present invention. See, Mead, C., Analog VLSI and Neural Systems, *Addison-Wesley* (1989). The circuitry used was CMOS (complimentary metal-oxide silicon) based and is also well known to those of ordinary skill in the art.

The chips were controlled by at least one Advantech PCL-812 digital I/O card (in the computer) that was driven by a Pentium based personal computer. These digital I/O cards can be obtained from Cyber Research, Branford, Connecticut. Preferably the chip is connected through interface hardware, i.e., an interface card, to the I/O card. The software for driving the I/O card can easily be written by one of ordinary skill in the art. DC voltage for powering the chips was provided by the PCL-812 and/or a Hewlett-Packard E3612A DC power supply. Voltage for the electrodes was supplied by the PCL-812 card and/or by an external Keithley 2400 source-measure unit.

The electrode array chips were designed so that the bond pads for all of the on-chip circuitry were located at one end of the long side of the chips. See **FIGURES 18a** and **18b**. The chips were attached to a standard 121 pin PGA (pin grid array) package that had been sawn in half so that approximately 2 cm of the chip extended out from the end, analogous to a diving board. See **FIGURE 18b**. PGA packages can be obtained from Spectrum Semiconductor

Materials, San Jose, California. Connecting wires ran between the bond pads on the chip and the contacts (bond pads) on the PGA package. The bond pads on the chip, the connecting wires, and the contacts on the PGA package were covered with epoxy for protection and insulation. See cut away in **FIGURE 18a**. The section of the chips that extended into the air contained the electrode array and was not covered by epoxy. This section of the chips was available for dipping into solutions of interest for chemical synthesis at the electrodes at the surface of the chip. One of ordinary skill in the art could easily set up and design chips appropriate for use in accordance with the present invention.

EXAMPLE 3 (Inventive) - Deprotection and Localization

Background Description

One of the above described electrode array chips comprising 16x64 platinum electrodes was used for this example. As indicated above, the chip contained 13 hardwired electrodes located at one end of the long side of the chip, however, these hardwired electrodes were not involved in this example.

The model chemical system used in this example to demonstrate localization and selective deprotection using electrochemically generated reagents involved attaching fluorescent labeled streptavidin molecules, a well-known variety of avidin, obtainable from Vector Laboratories, Burlingame, California, to a membrane overlaying the electrode array chip via a trityl linker molecule. The overlaying membrane used was polysaccharide-based. The trityl linker molecule used was acid labile, i.e., labile to protons, and detached from the overlaying membrane in the presence of protons, taking with it the attached fluorescent labeled streptavidin molecule. More specifically, the trityl linker molecule used was a modified 4,4'dimethoxytrityl molecule with an exocyclic active ester obtained from Perseptive Biosystems, Framingham, Massachusetts.

Experimental Procedure

Preparation of the chip for attachment of molecules

To enable the attachment of molecules, in particular trityl linker molecules, to the surface of the electrode array chip for synthesis and/or deprotection proximate the electrodes, the chip was coated/modified with an overlaying membrane of a polysaccharide-based material. Specifically, a polygalactoside was used as the overlaying membrane material in this example. The polygalactoside membrane was dip coated onto the chip.

Attachment of the trityl linker molecules

Once the electrode array chip was coated with the polysaccharide membrane, the trityl linker molecules were attached to the chip. The trityl linker molecule used for this example was a modified 4,4'-dimethoxytrityl molecule with an exocyclic active ester, specifically the molecule was N-succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate. The synthesis and use of this molecule is described in *A Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules*, by Brian D. Gildea, James M. Coull and Hubert Koester, Tetrahedron Letters, Volume 31, No. 49, pgs 7095-7098 (1990).

The trityl linker molecules were attached to the polysaccharide membrane via immersion of the polysaccharide membrane coated chip in a DMF solution containing 0.5M of tertbutyl ammonium perchlorate, 0.75M of 2,4,6-collidine and 0.2M of the trityl linker. The immersion of the polysaccharide membrane coated chip in the DMF linker solution lasted for 30 minutes at ambient temperature. However, dipping or coating according to any method known to one of ordinary skill in the art would be acceptable.
The trityl linker coated chip was then washed with DMF to remove any remaining reactants. Next, the trityl linker coated chip was washed in an aqueous 0.1M sodium phosphate buffer that was adjusted to pH 8.0, and dried.

Attachment of the fluorescent dye labeled molecules

The trityl linker coated chip was then immersed in an aqueous solution of fluorescent dye (Texas Red) labeled streptavidin molecules having a concentration of 50 micrograms per milliliter and allowed to remain in this

solution for one hour at ambient temperature. During this immersion, the linker molecule was derivatized and the fluorescent dye labeled streptavidin molecules were attached to the linker molecules.

The chip containing fluorescent dye labeled streptavidin molecules was then washed with an aqueous 0.1M sodium phosphate buffer that was adjusted to pH 8.0 to remove remaining reactants, and dried. The chip was now ready for use in the electrochemical process of the invention, i.e., the selective deprotection step.

Following exposure of the prepared chip to the fluorescent labeled streptavidin molecules, but prior to any electrical current or voltage being applied. the electrodes in the array were all bright with fluorescence because the membrane proximate to them contained the fluorescent labeled streptavidin molecules bound to the membrane via the trityl linker. A photomicrograph of this is shown in **FIGURE 19a**.

Selective Deprotection

To perform the selective deprotection step, the prepared chip was immersed in a 0.05M aqueous sodium phosphate buffer solution to enable electrochemical generation of reagents. A voltage difference of 2.8 volts was applied to select electrodes (alternating in a checkerboard pattern) for approximately 10 minutes, causing protons to be generated electrochemically at the anodes.

After the protons were electrochemically generated at the anodes, the anodes became dark because the trityl linker previously bound proximate to the anodes dissociated from the anodes and the fluorescent labeled streptavidin molecules were washed away. The extent to which this occurred at the anodes and not at the cathodes in the checkerboard pattern, is a measure of the chemical crosstalk occurring between the electrodes in the array. That is, if chemical crosstalk were occurring, the cathodes would also be dark because the protons would have migrated and dissociated the trityl linkers at the cathodes.

Thus, under epifluorescent microscopy, the bright electrodes (cathodes) indicate the presence of a Texas Red labeled streptavidin molecule bound to a linker molecule at the electrode and the dark electrodes (anodes) indicate the lack of a Texas Red labeled streptavidin molecule bound to a linker molecule at the electrode. This is shown in **FIGURES 20** and **21**, **FIGURE 20** having been taken using a 4x objective with an integration time of 2 seconds, and **FIGURE 21** having been taken using a 10x objective with a 500 millisecond integration time.

Results

Following drying of the chip, photomicrographs were taken of the electrode array following completion of the deprotection step, and are reproduced in **FIGURES 20 and 21**. As shown in these figures, selective deprotection was achieved using the process of the present invention. As is shown in these figures, a repeating checkerboard pattern was produced, exemplifying that the process of the present invention achieved localization of the protons generated at the anodes and prevented migration of these protons to the cathodes. The dark areas (anodes) are clearly defined and distinguished from the also clearly defined bright areas (cathodes). The clearly demarcated checkerboard pattern shown in the photomicrographs indicates that no, or very little, chemical cross talk occurred during the deprotection step.

EXAMPLE 4 - Comparative Example

Using two electrode array chips prepared in accordance with the present invention, one chip was processed using the selective deprotection procedure in accordance with the present invention using a buffering solution, and the second chip was processed using a selective deprotection procedure varying only in that the electrolyte used in the Examples of Southern (WO 93/22480, filed November 11, 1993) replaced the buffering solution of the present invention.

Rather than using an electrode array, this comparison was conducted on a few of the hard wired electrodes found on the side of the electrode array

chips. **FIGURE 17** is a photomicrograph taken under the same conditions as FIGURE 14, but showing the hard wired electrodes used in this example.

Deprotection in accordance with the invention

The steps of coating the chip with the polysaccharide membrane and attaching the trityl linker molecules to the membrane were performed in accordance with the procedures used above in Example 3.

The attaching of the fluorescent dye labeled streptavidin molecules and the deprotection steps were also performed in accordance with Example 3, but a 20 mM aqueous sodium phosphate buffer solution was used instead of the 0.05M solution used in Example 3, to enable the electrochemical generation of reagents. The voltage that was applied between selected electrodes was 2.8 volts, which was applied for approximately 30 seconds.

Similar results to Example 3 were obtained. These results are shown in **FIGURES 22-24**.

FIGURE 22 shows the hardwired electrodes involved in this process, labeled as T1, T2 and T4. In this process, T1 was the counter electrode, i.e., the cathode, and T2 and T4 were the anodes where protons were generated upon the application of the electric current or voltage. No voltage had been applied to the electrodes shown in **FIGURE 22**.

FIGURE 23 shows the same electrodes following derivatization or bonding with the fluorescent labeled streptavidin molecules. As is shown, electrodes T2 and T4 are bright, indicating the presence of a Texas Red labeled streptavidin molecule bound to a linker molecule proximate each of these electrodes.

FIGURE 24 shows the condition of anodes T2 and T4 following application of the voltage causing electrochemical generation of protons at the anodes and resultant dissociation of the trityl linker at these positions. Once dissociation occurred, the fluorescent labeled streptavidin molecules were washed away, leaving the anodes dark. Notably, anodes T2 and T4 are darker than the neighboring electrodes, indicating no chemical crosstalk was occurring.

As is shown by **FIGURES 23 and 24**, localization and selective deprotection were achieved at anodes T2 and T4, as was desired.

Deprotection using electrolyte of Southern (WO 93/24480)

All steps were performed identical to that for the above process in accordance with the present invention, except that instead of using a buffering solution in accordance with the invention, deprotection was performed in the presence of a 1% triethylammonium sulfate electrolyte in an acetonitrile solvent, as disclosed in the Examples of Southern.

The results of this process are shown in **FIGURES 25a, 25b, 26a** and **26b**.

In the electrodes shown, labeled T1 and T4, electrode T1 represented the cathode and electrode T4 represented the anode.

FIGURES 25a, 25b, 26a and **26b** show that the membrane exhibited random and imprecise bright and dark areas. These bright and dark areas indicate that the protons generated at the anode (electrode T4) are not confined or localized to the area proximate the electrode, causing significant dissociation of the trityl linker over the entire field of the photomicrograph. T1 appears to have retained most of the fluorescence directly above the electrode. This is explained by the base that is generated at the T1 cathode, which neutralized the acid generated proximate the T4 anode.

As is seen from a comparison of the photomicrographs illustrating the results achieved in accordance with the present invention (i.e., using a buffering solution overlaying the electrodes) and those illustrating the results achieved from the analogous experiment performed using the electrolyte of Southern (WO 93/22480), superior localization of the electrochemical generated reagents was achieved using the process of the present invention. The superior localization achieved in accordance with the present invention greatly reduced, if not eliminated, undesirable chemical crosstalk between proximate electrodes. In contrast, very little localization of the electrochemical generated reagents was achieved using the electrolyte of the prior art, resulting in random and imprecise deprotection over the entire field of the micrograph.

What is claimed is:

1. A method for electrochemical placement of a material at a specific location on a substrate, which comprises the steps of:

providing a substrate having at its surface at least one electrode that is proximate to at least one molecule bearing at least one protected chemical functional group,

applying a potential to said electrode sufficient to generate electrochemical reagents capable of deprotecting at least one of the protected chemical functional groups of said molecule, and

bonding the deprotected chemical functional group with a monomer or a pre-formed molecule.

2. A method according to claim 1, further comprising placing a buffering or scavenging solution in contact with the electrode at the surface of the substrate to prevent the electrochemically generated reagents from leaving the locality of the electrode.

3. A method according to claim 2, wherein said buffering solution is selected from acetate buffers, borate buffers, carbonate buffers, citrate buffers, glycine buffers, HEPES buffers, MOPS buffers, phosphate buffers, TRIS buffers and KI solutions.

4. A method according to claim 2, wherein said buffering solution is present in a concentration of at least 0.01 mM.

5. A method according to claim 2, wherein the concentration of the buffering solution ranges from 0.1 to 100 mM.

6. A method according to claim 1, wherein said monomer or pre-formed molecule has at least one other protected chemical functional group at a site different from where bonding with the deprotected chemical functional group of the molecule occurs.

7. A method according to claim 1, wherein said monomer is an amino acid.

8. A method according to claim 1, wherein said pre-formed molecule is selected from proteins, nucleic acids, polysaccharides, and porphyrins.

9. A method according to claim 1, wherein said molecule is a linker molecule or a monomer.

10. A method according to claim 1, wherein said molecule is directly attached to the surface of said substrate, is attached to the surface of said substrate via a linker molecule, or is attached to a layer of material overlaying said substrate.

11. A method according to claim 9, wherein said linker molecule comprises a group cleavable by an electrochemically generated reagent, which cleavable group enables removal of the material from the substrate.

12. A method according to claim 1, wherein said protected chemical functional groups are protected with an acid or base labile protecting group.

13. A method according to claim 1, wherein said at least one electrode comprises an array of electrodes.

14. A method according to claim 13, wherein said array of electrodes comprises at least 100 electrodes.

15. A method according to claim 6, further comprising sequentially deprotecting the other protected chemical functional group of the monomer or pre-formed molecule and bonding to the deprotected monomer or pre-formed molecule another monomer or pre-formed molecule.

16. A method for electrochemical synthesis of an array of separately formed polymers on a substrate, which comprises the steps of:

placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more molecules bearing at least one protected chemical functional group attached thereto,

selectively deprotecting at least one protected chemical functional group on at least one of said molecules;

bonding a first monomer having at least one protected chemical functional group to one or more deprotected chemical functional groups of said molecule;

selectively deprotecting a chemical functional group on the bonded molecule or another of said molecules bearing at least one protected chemical functional group;

bonding a second monomer having at least one protected chemical functional group to a deprotected chemical functional group of the bonded molecule or said other deprotected molecule; and

repeating the selective deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule and the subsequent bonding of an additional monomer to said deprotected chemical functional group until at least two separate polymers of desired length are formed on the substrate surface.

17. A method according to claim 16, wherein during said selective deprotection steps, an electric potential is applied to one or more selected electrodes sufficient to generate electrochemical reagents at the selected electrodes capable of deprotecting the chemical functional groups on said proximate molecules or monomers.

18. A method according to claim 16, wherein said buffering or scavenging solution prevents the electrochemical reagents generated at selected electrodes from deprotecting the chemical functional groups of molecules or monomers proximate to unselected electrodes.

19. A method according to claim 16, wherein said buffering solution is selected from acetate buffers, borate buffers, carbonate buffers, citrate buffers, glycine buffers, HEPES buffers, MOPS buffers, phosphate buffers, TRIS buffers and KI solutions.

20. A method according to claim 16, wherein said buffering solution is present in a concentration of at least 0.01 mM.

21. A method according to claim 16, wherein the concentration of the buffering solution ranges from 0.1 to 100 mM.

22. A method according to claim 16, wherein said monomers are amino acids.

23. A method according to claim 16, wherein said molecules are linker molecules or monomers.

24. A method according to claim 16, wherein said molecules are directly attached to the substrate surface, are attached to the substrate surface via a linker molecule, or are attached to a layer of material overlaying said substrate surface.

25. A method according to claim 24, wherein said overlaying layer is controlled porosity glass.

26. A method according to claim 23, wherein said linker molecule comprises a group cleavable by an electrochemically generated reagent, which cleavable group enables removal from said substrate of one or more bonded molecules.

27. A method according to claim 16, wherein said protected chemical functional groups are protected with an acid or base labile protecting group.

28. A method according to claim 16, wherein said substrate is formed from at least one material selected from undoped semiconductors, glass, ceramics, polymers, and waxes.

29. A method according to claim 16, wherein said array of electrodes comprises at least 100 electrodes.

30. A method according to claim 16, wherein said array of electrodes comprises a matrix having at least 2048 electrodes.

31. A method according to claim 30, wherein said array of electrodes comprises a matrix having at least 204,800 electrodes.

32. A method according to claim 16, wherein each of the electrodes in said array ranges in diameter from less than 1 micron to about 100 microns.

33. A method according to claim 16, wherein the electrodes of said array are formed from platinum or palladium.

34. A method according to claim 33, wherein said platinum or palladium electrodes are preloaded with hydrogen.

35. A method according to claim 16, which further comprises a capping step wherein unbonded deprotected chemical functional groups on said molecules or monomers are capped with acetic anhydride or n-acetylimidizole.

36. A method according to claim 16, which further comprises an additional bonding step wherein a pre-formed molecule is bonded to a deprotected chemical functional group on one or more of said molecules or monomers.

37. A method according to claim 36, wherein said pre-formed molecule is selected from proteins, nucleic acids, polysaccharides, and porphyrins.

38. A method according to claim 36, wherein said pre-formed molecule bears at least one protected chemical functional group to which an additional monomer may bond following selective deprotection of the chemical functional group on the pre-formed molecule.

39. A method according to claim 17, wherein the one or more selected electrodes to which an electric potential is applied are selected by at a switching mechanism selected from CMOS switching circuitry, radio frequency addressable switches, microwave frequency addressable switches and light addressable switches.

40. A method according to claim 16, wherein said array of electrodes comprises at least 1024 electrodes.

41. A method for electrochemical synthesis of an array of separately formed oligonucleotides on a substrate, which comprises the steps of:

placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more molecules bearing at least one protected chemical functional group attached thereto,

selectively deprotecting at least one protected chemical functional group on at least one of said molecules;

bonding a first nucleotide having at least one protected chemical

functional group to one or more deprotected chemical functional groups of said molecule;

selectively deprotecting a chemical functional group on the nucleotide bonded molecule or another of said molecules bearing at least one protected chemical functional group;

bonding a second nucleotide having at least one protected chemical functional group to a deprotected chemical functional group of the nucleotide bonded molecule or said other deprotected molecule; and

repeating the selective deprotection of a chemical functional group on a protected bonded nucleotide or a protected bonded molecule and the subsequent bonding of an additional nucleotide to said deprotected chemical functional group until at least two separate oligonucleotides of desired length are formed on the substrate surface.

42. A method according to claim 41, wherein during said selective deprotection steps, an electric potential is applied to one or more selected electrodes sufficient to generate electrochemical reagents at the selected electrodes capable of deprotecting the chemical functional groups on said proximate molecules or nucleic acids.

43. A method according to claim 41, wherein said buffering or scavenging solution prevents the electrochemical reagents generated at selected electrodes from deprotecting the chemical functional groups of molecules or nucleotides proximate to unselected electrodes.

44. A method according to claim 8, wherein said pre-formed molecule is a nucleic acid.

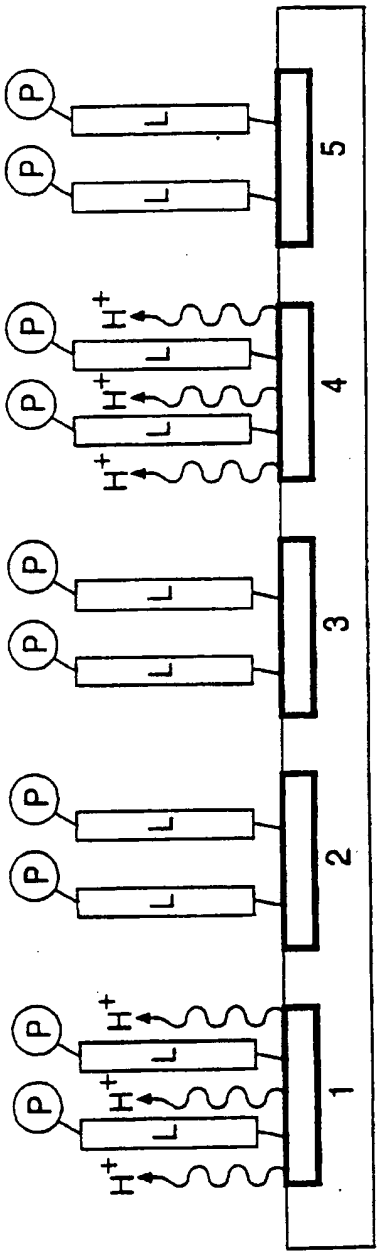

FIG. 1a

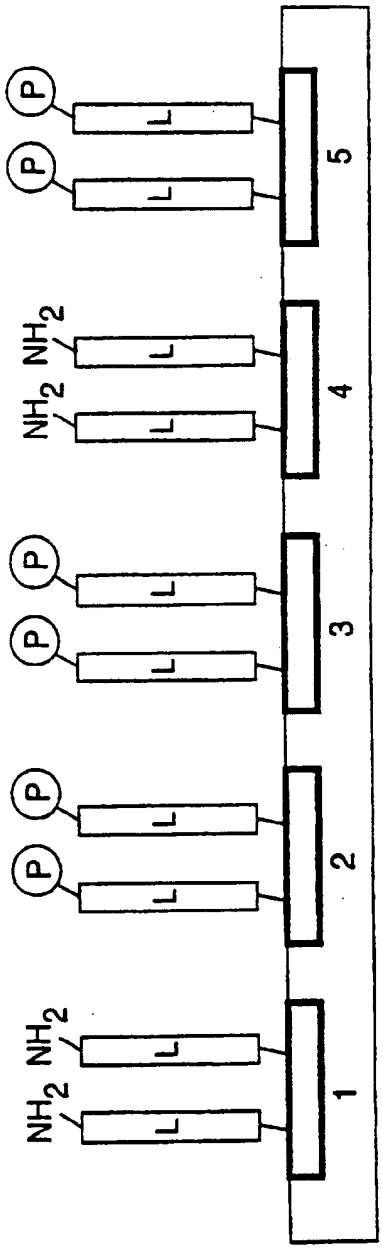

FIG. 1b

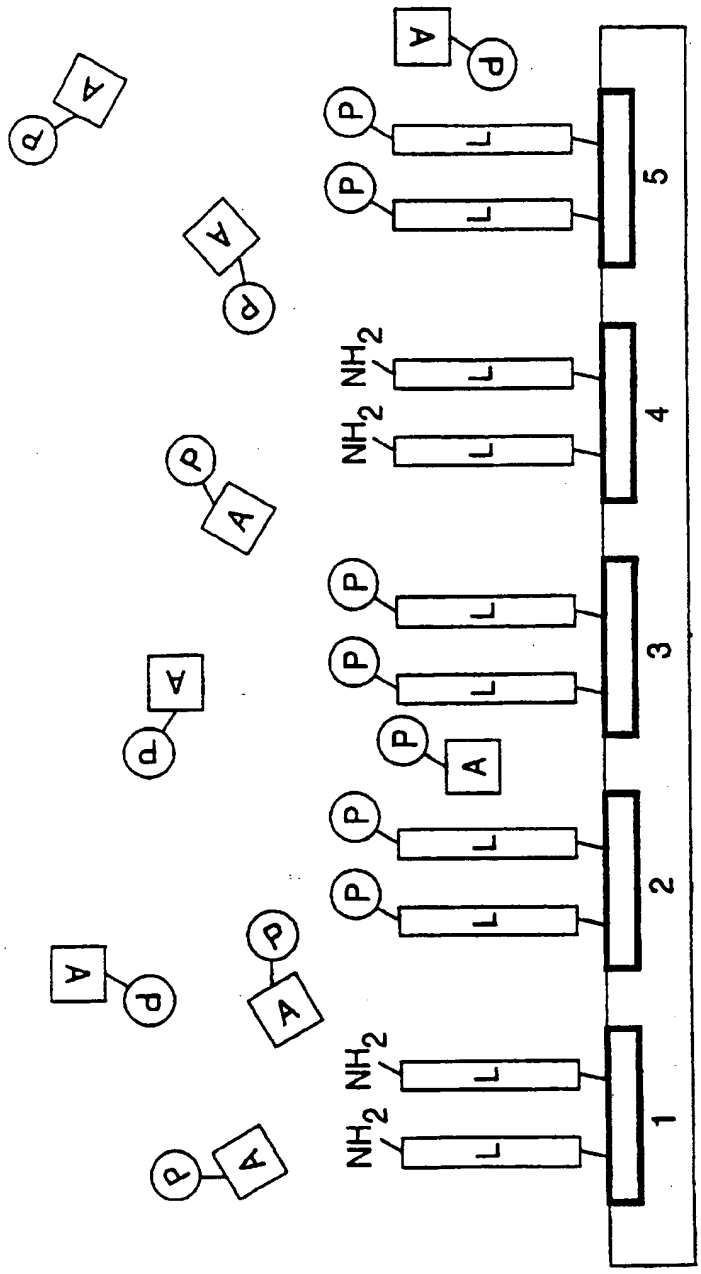

FIG. 2a

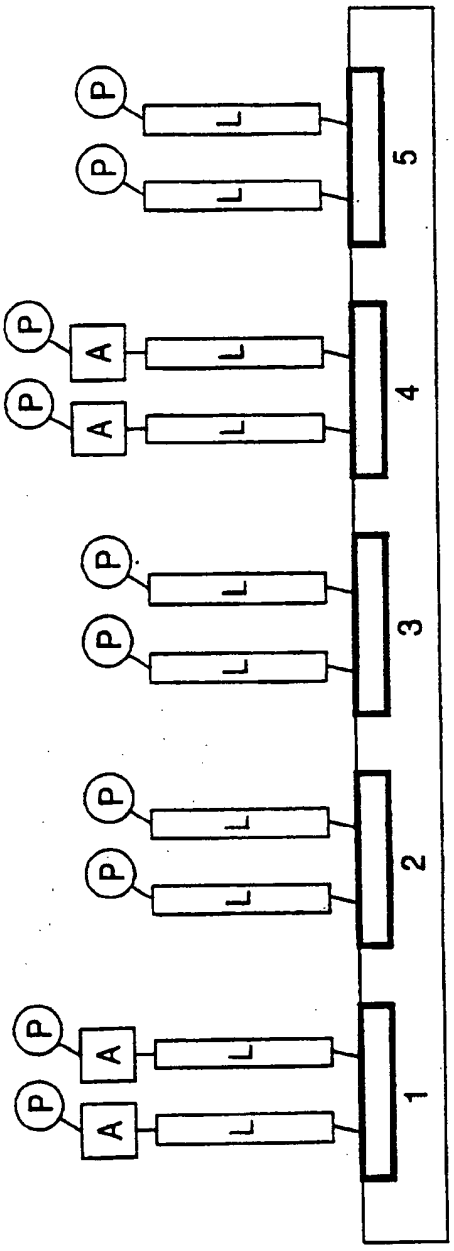

FIG. 2b

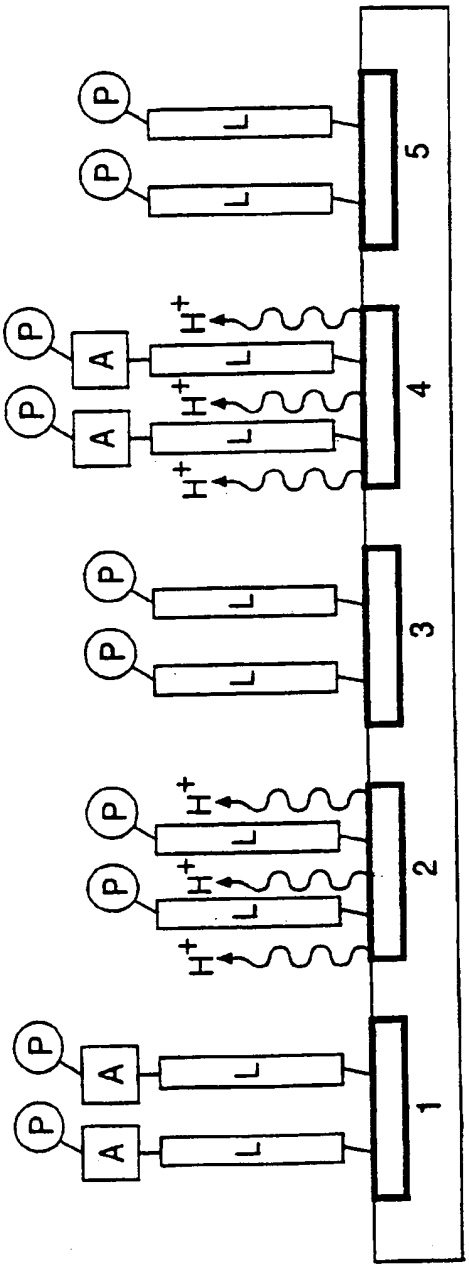

FIG. 3a

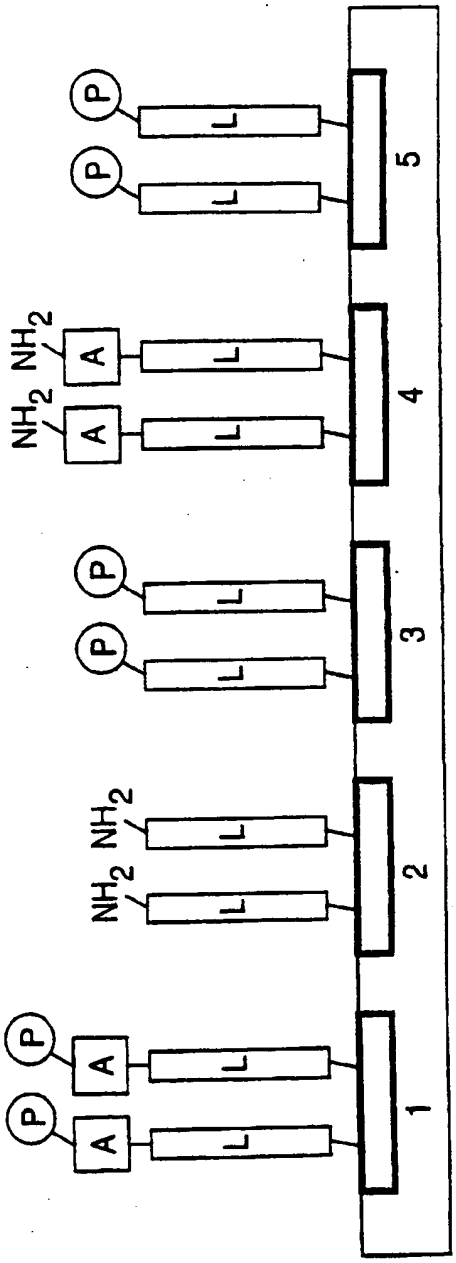

FIG. 3b

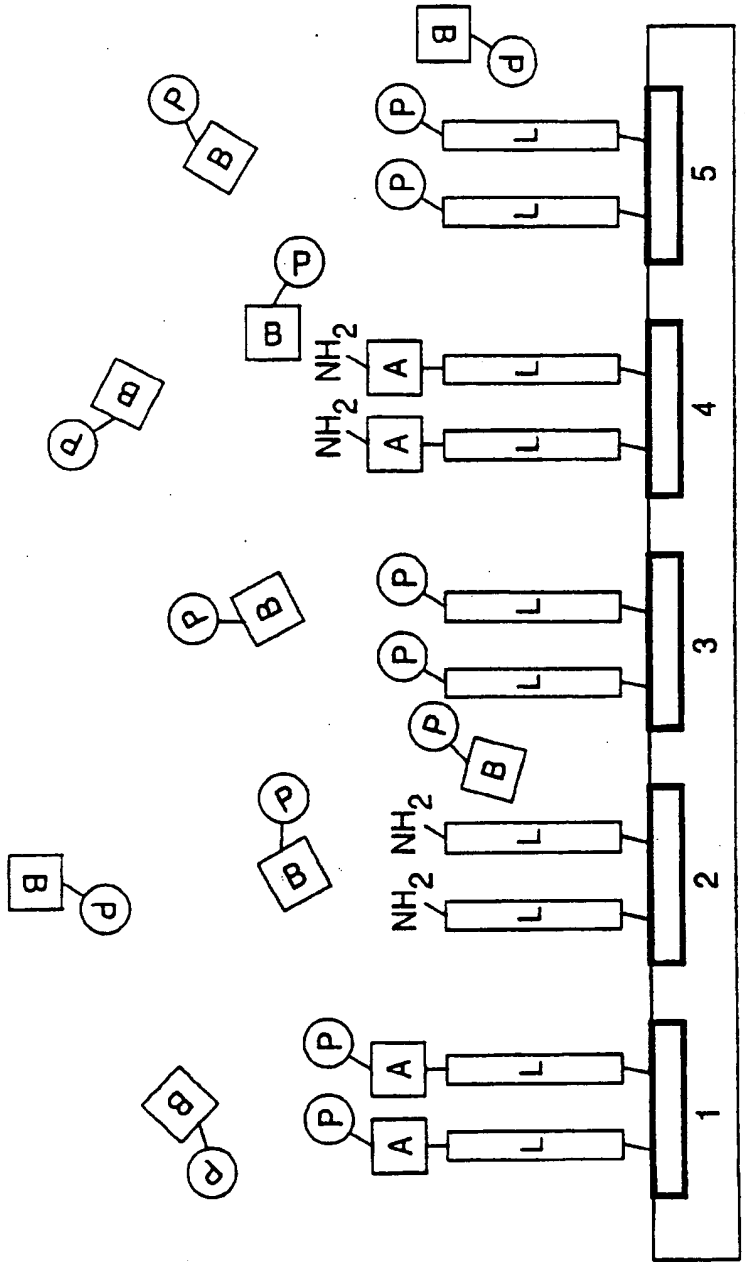

FIG. 4a

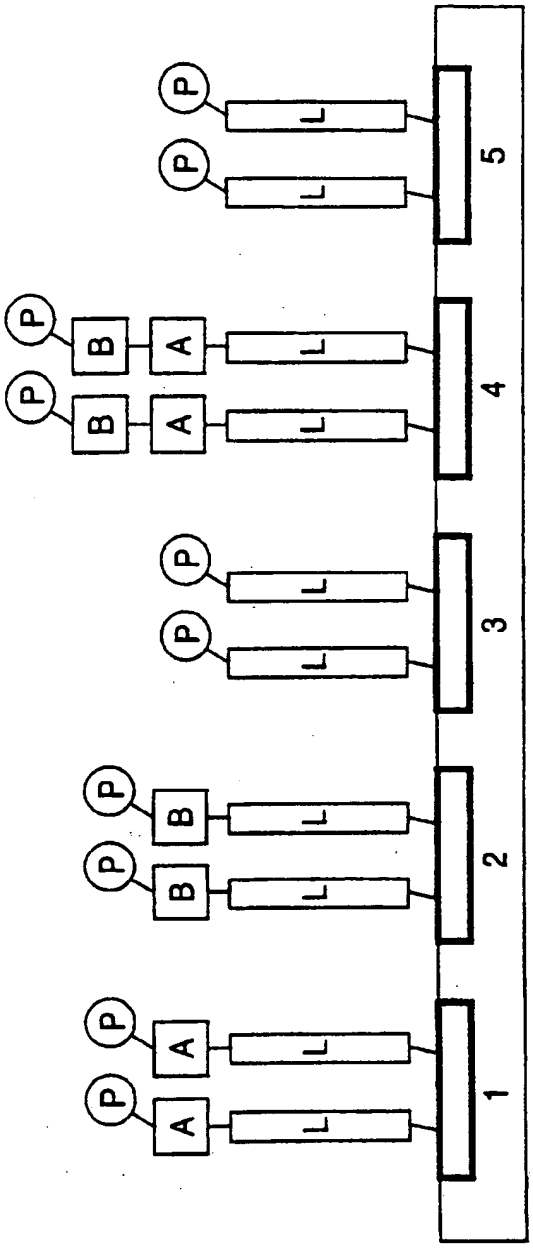

FIG. 4b

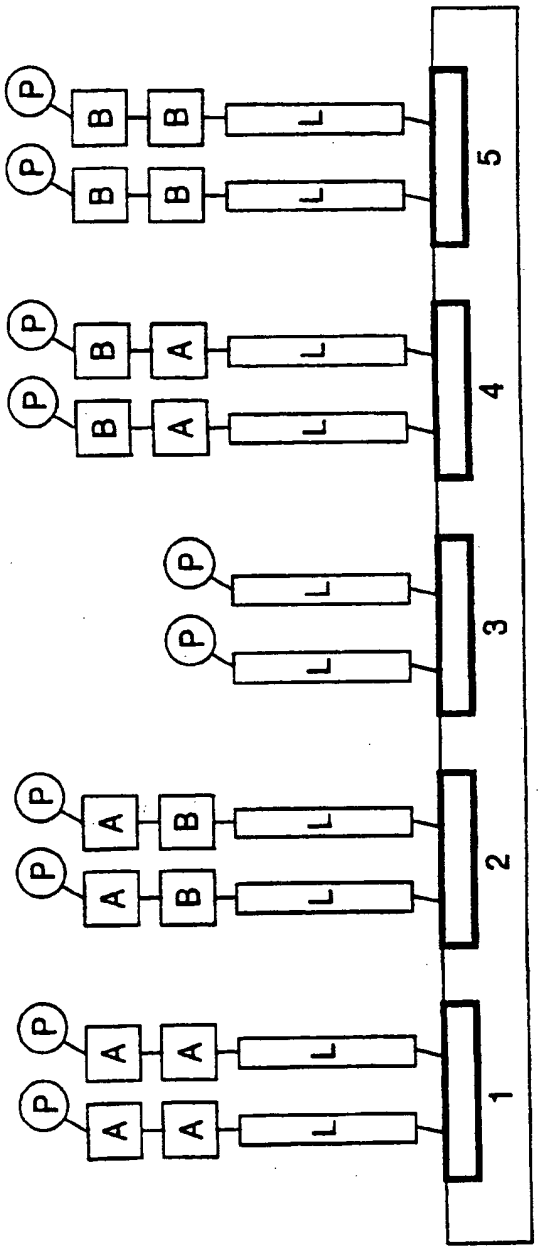

FIG. 5

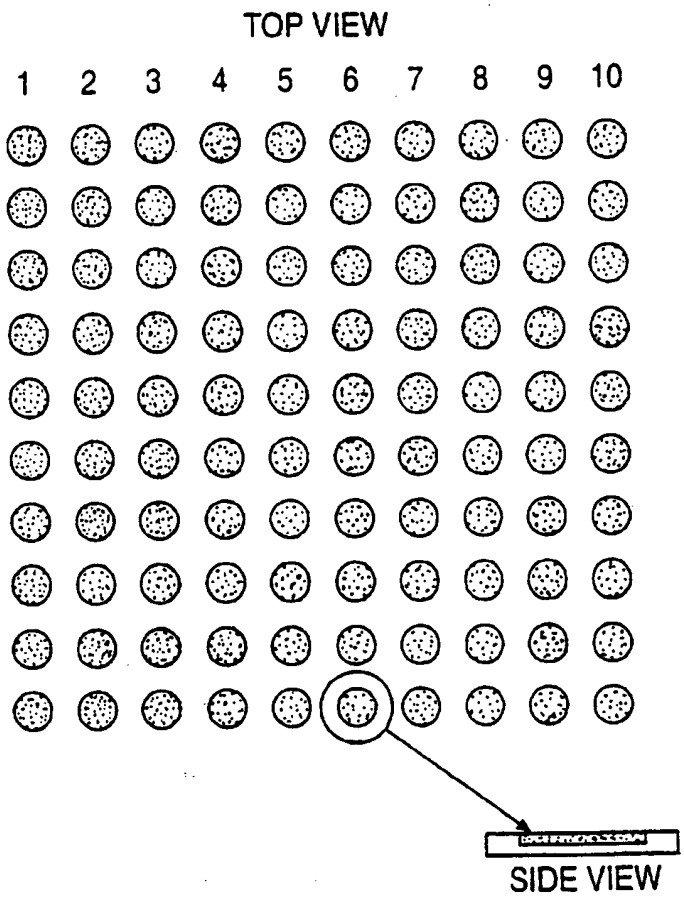

FIG. 6

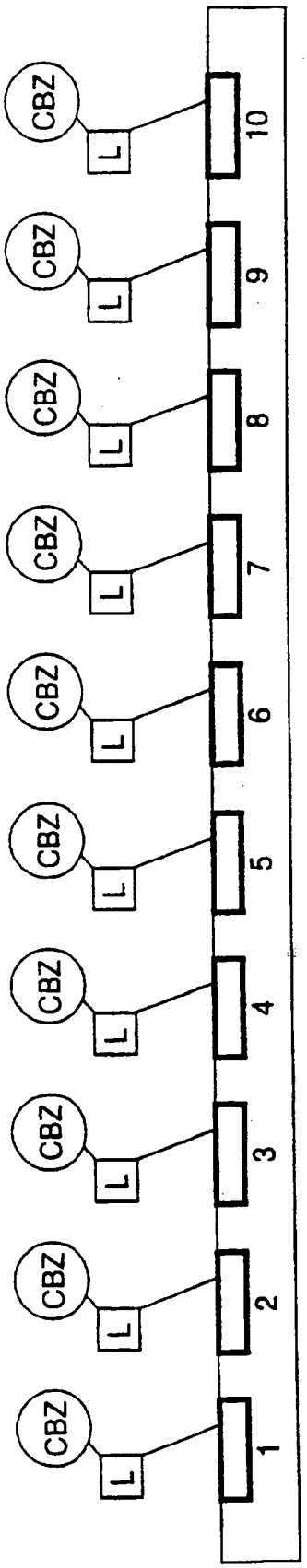

FIG. 7

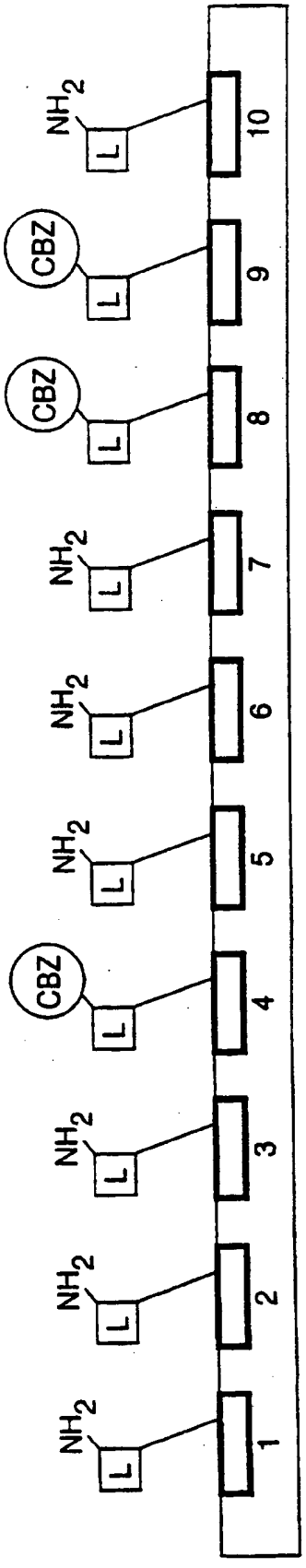

FIG. 8

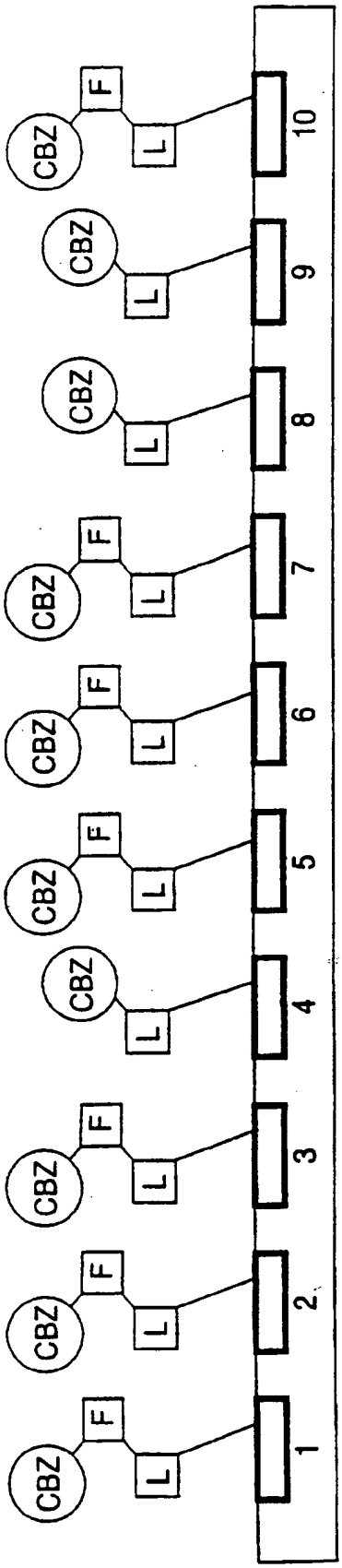

FIG. 9

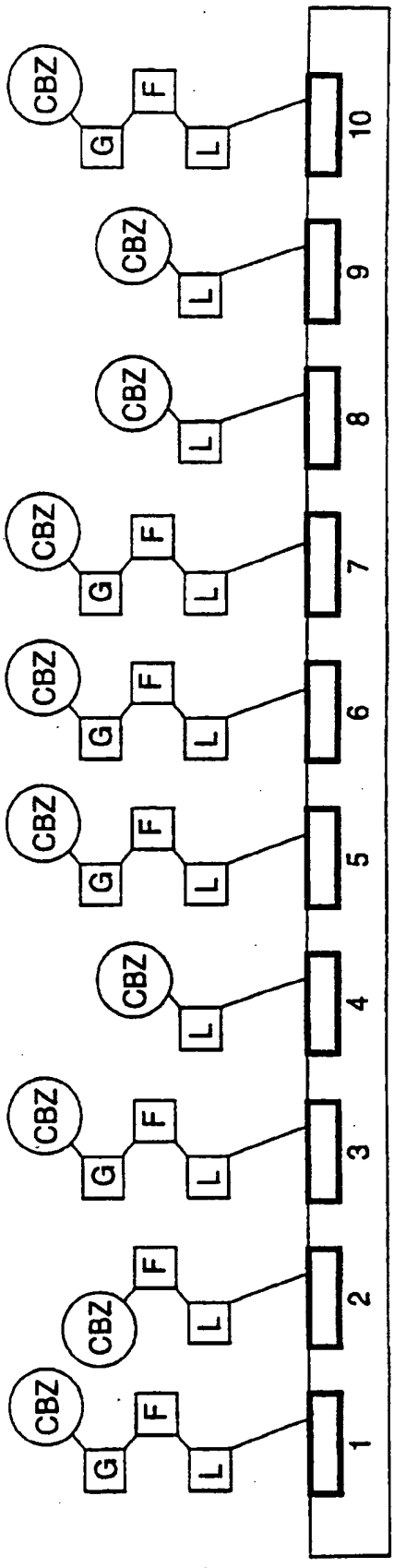

FIG. 10

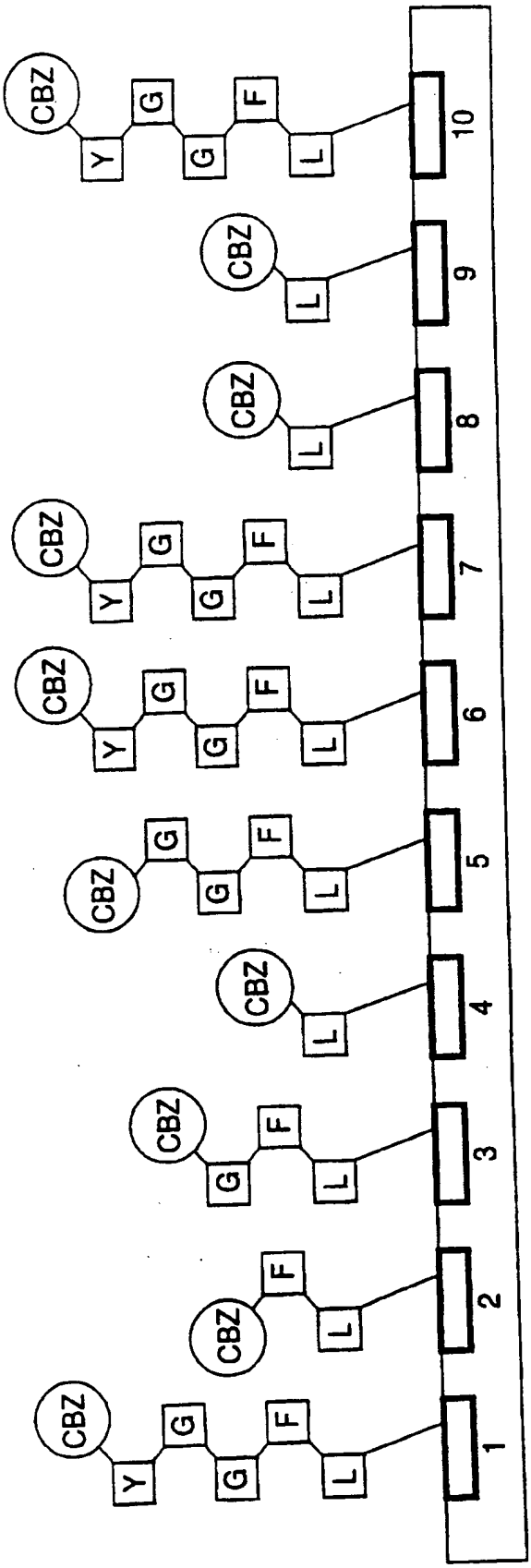
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FIG. 11

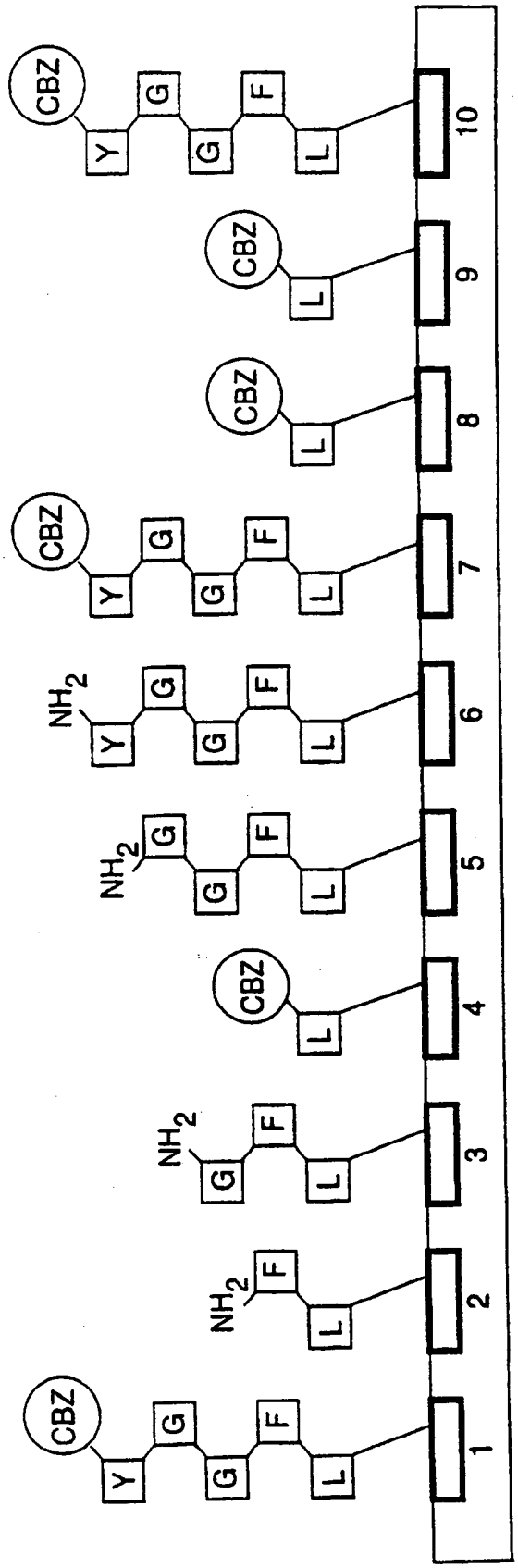
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FIG. 12

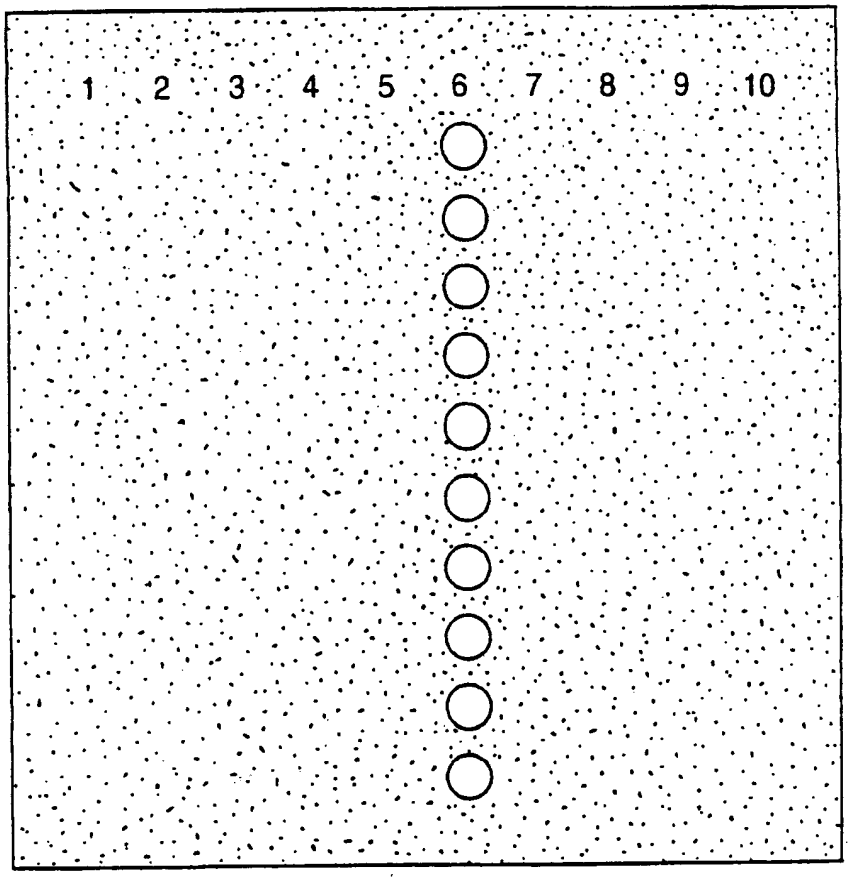

FIG. 13

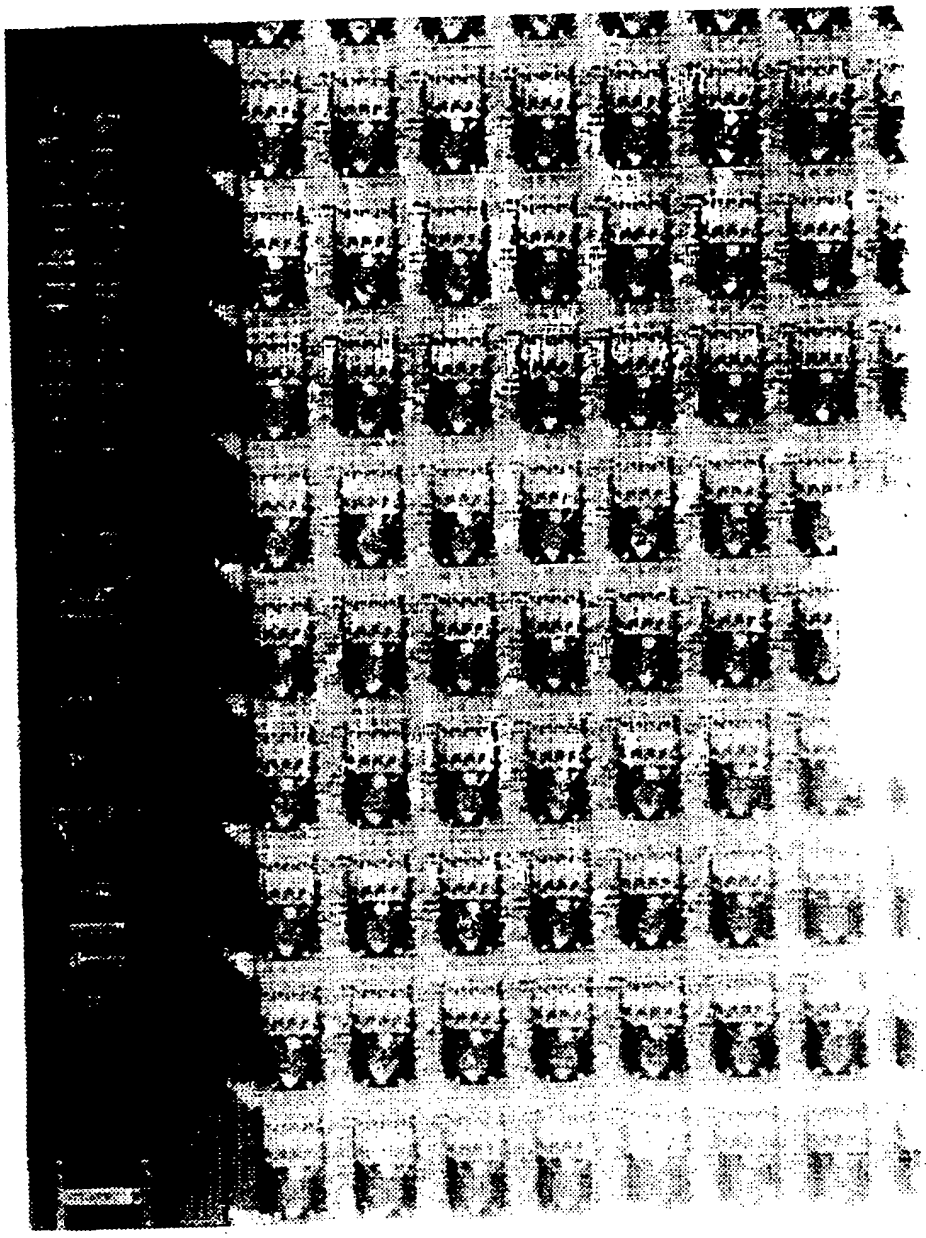

FIG. 14

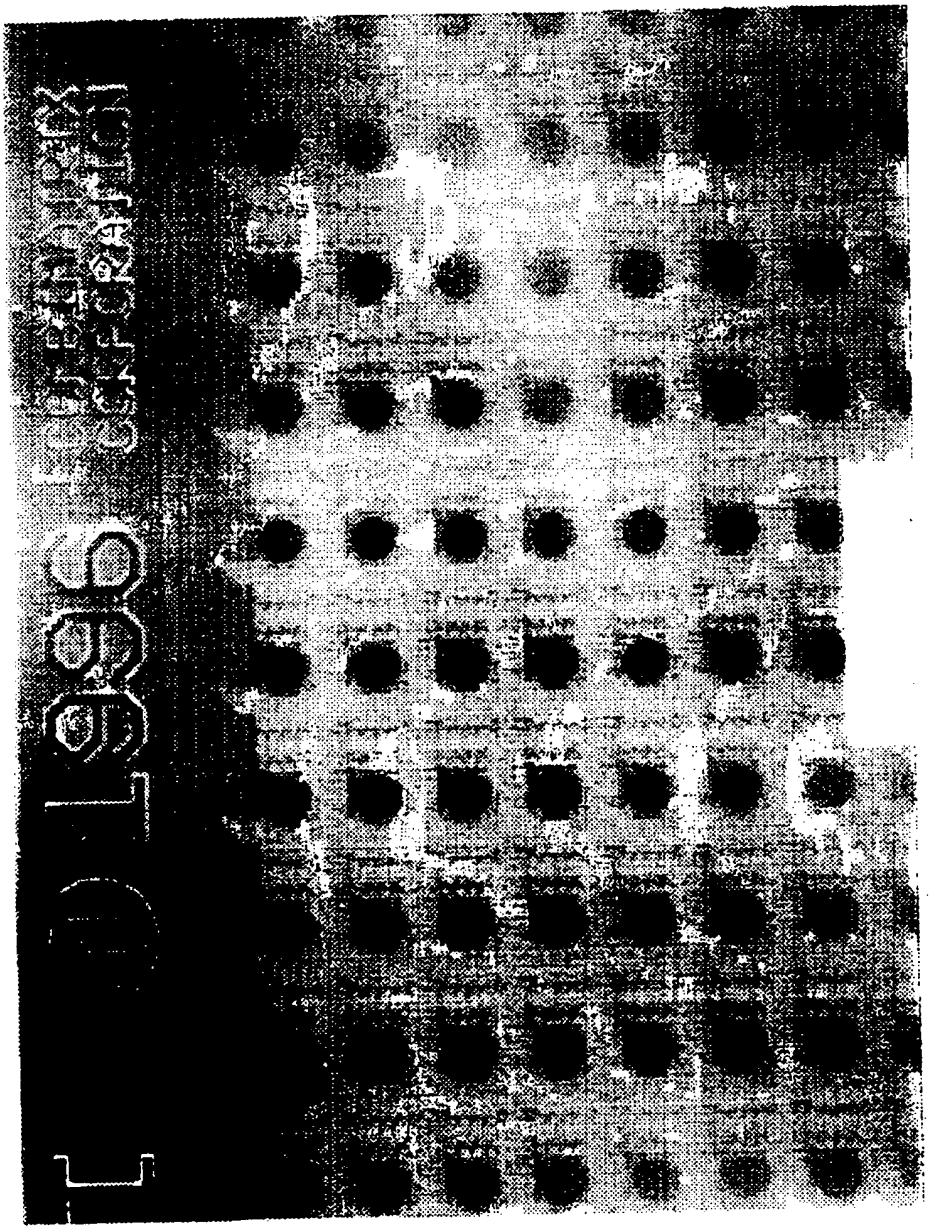

FIG. 15

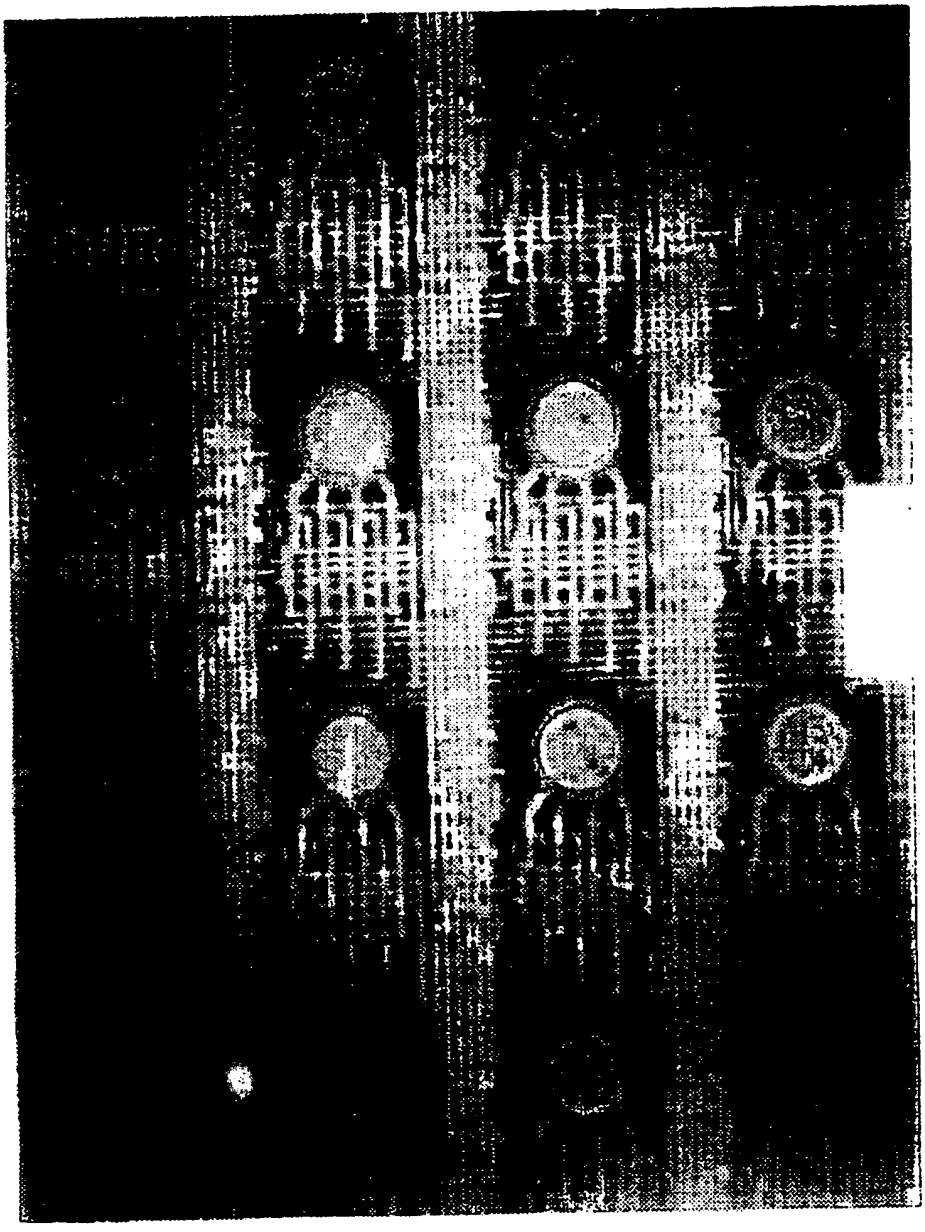

FIG. 16

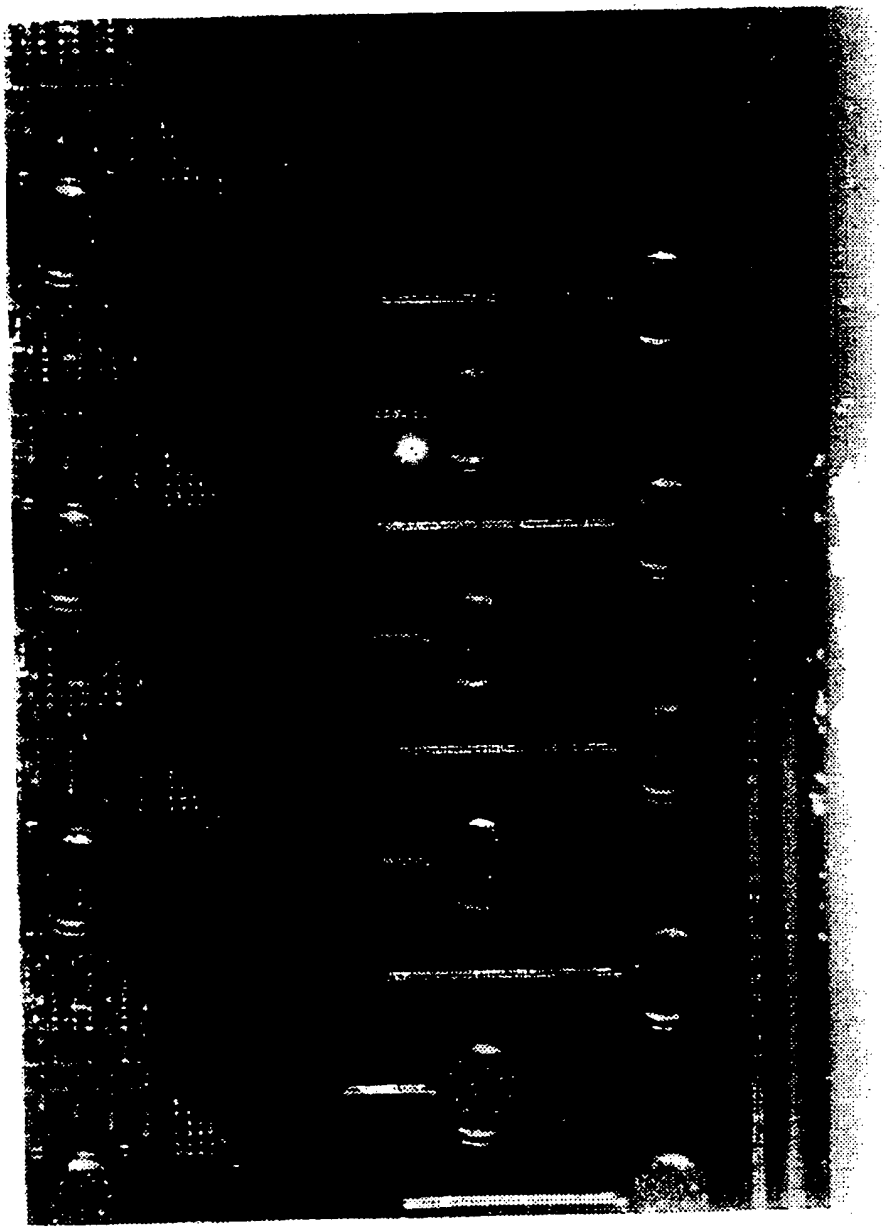

FIG. 17

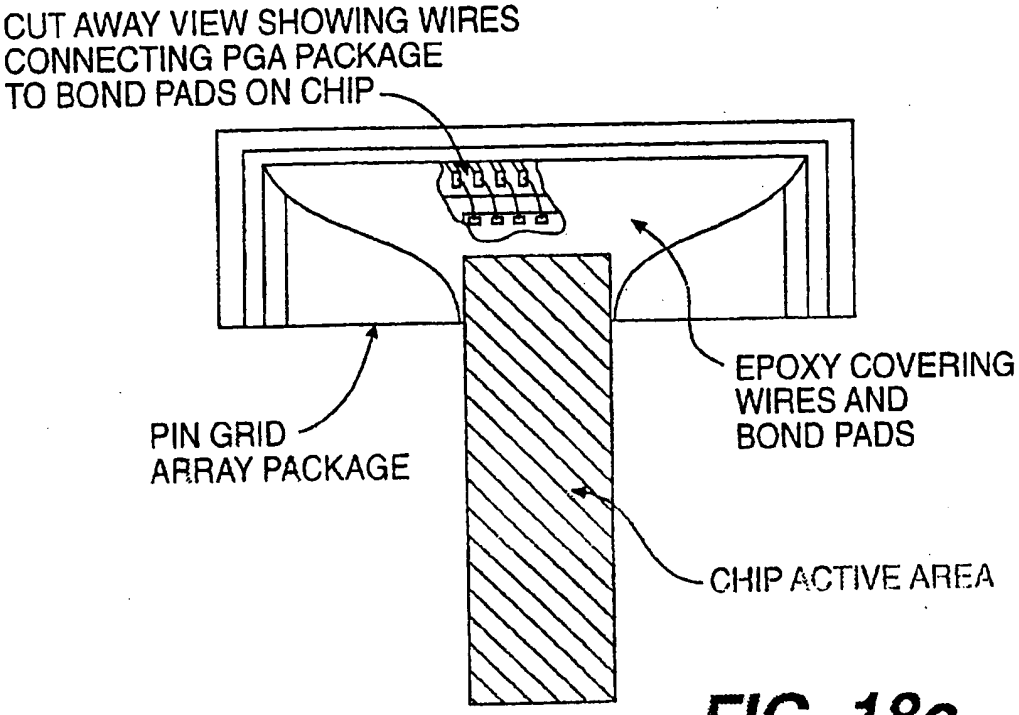

FIG. 18a

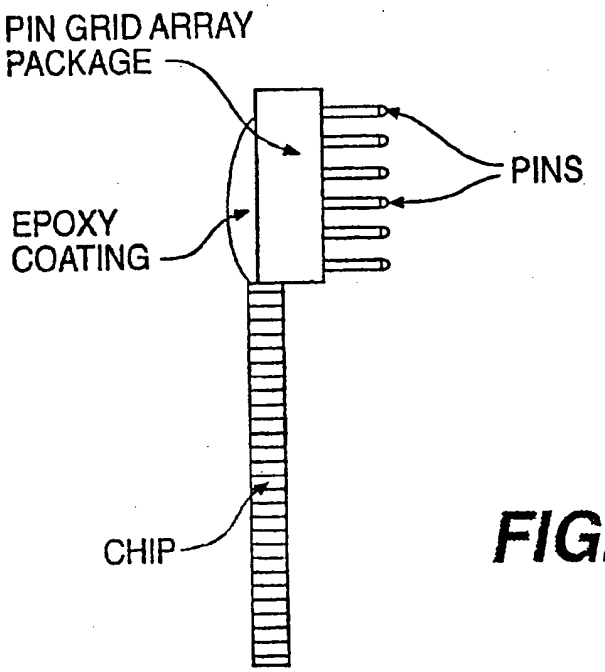

FIG. 18b

FIG. 19a

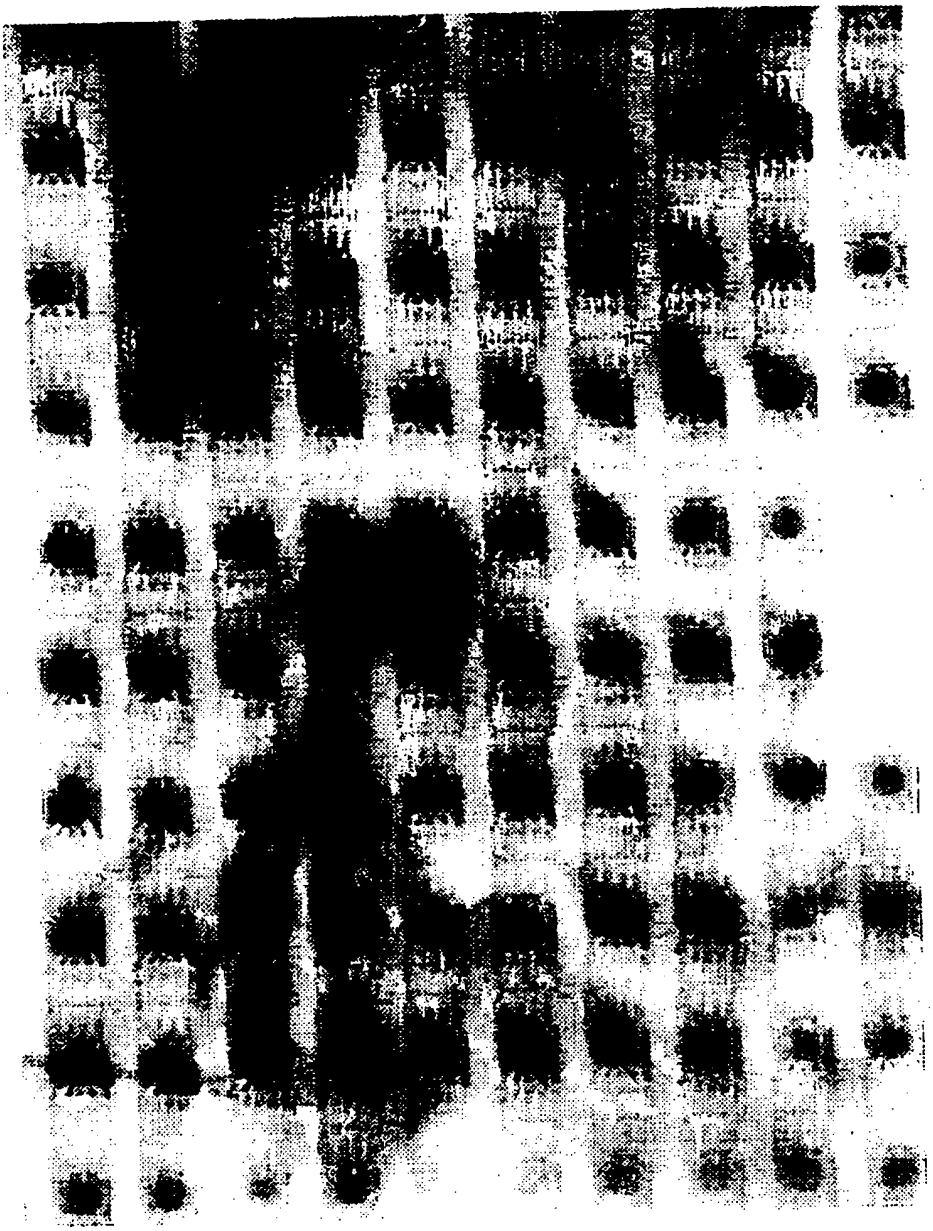

FIG. 19b

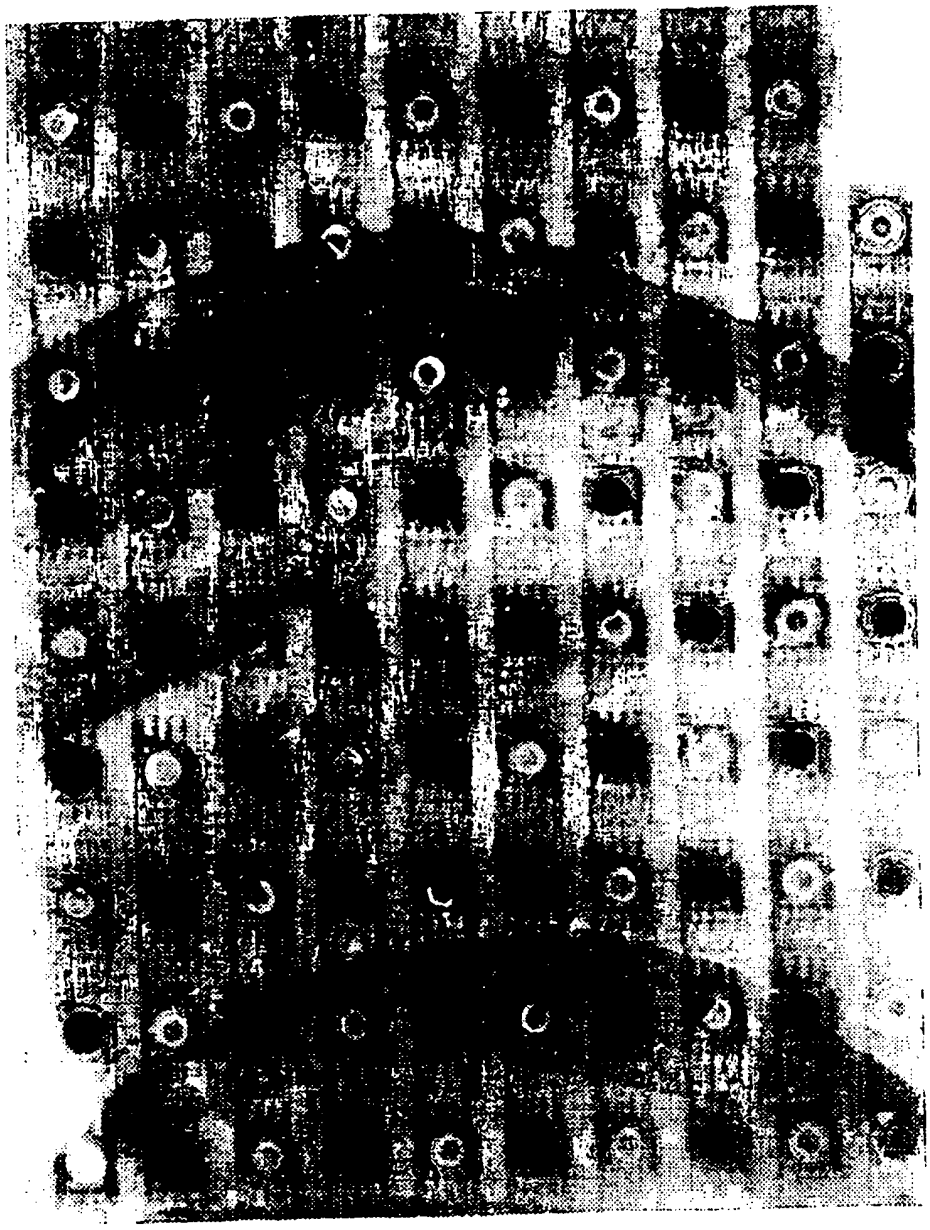

FIG. 20

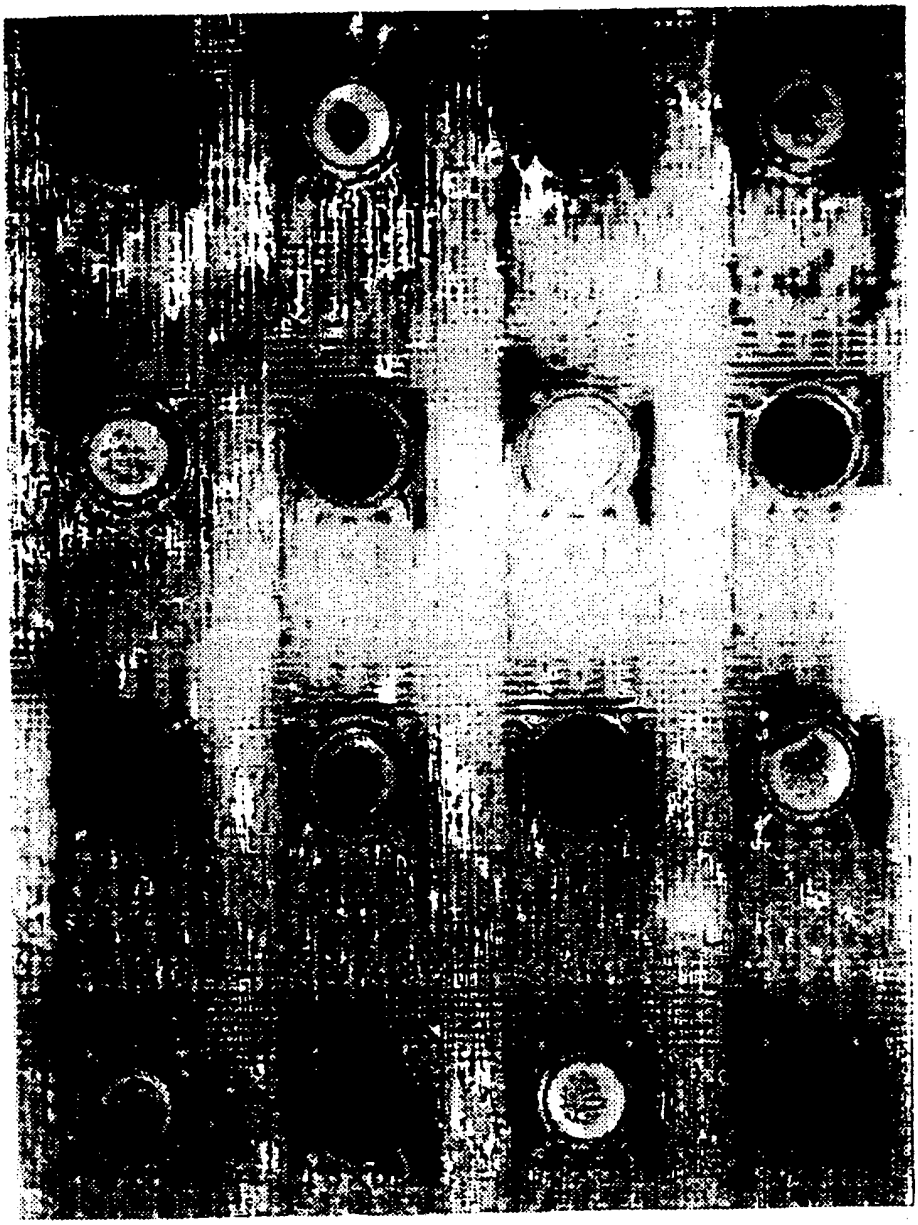

FIG. 21

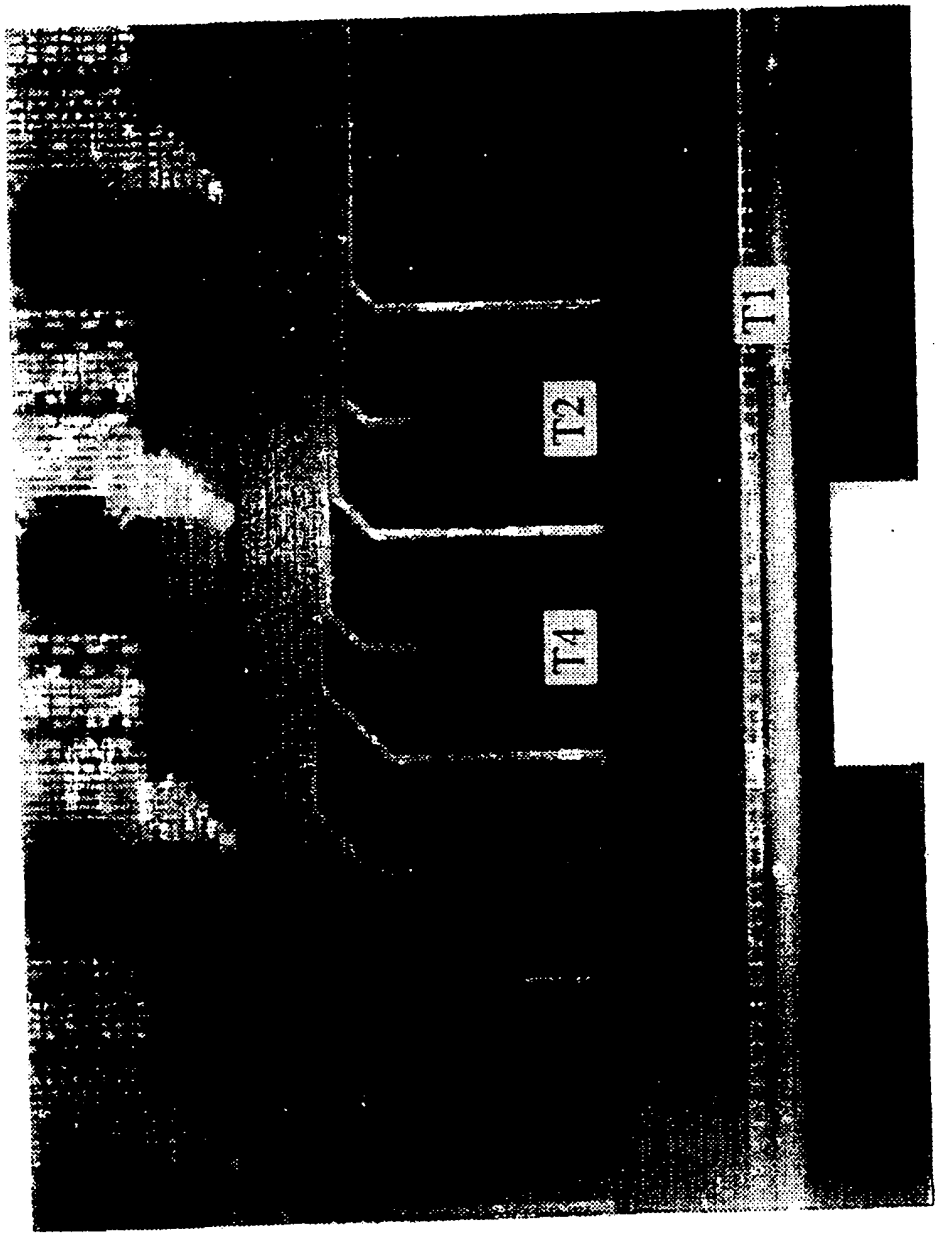

FIG. 22

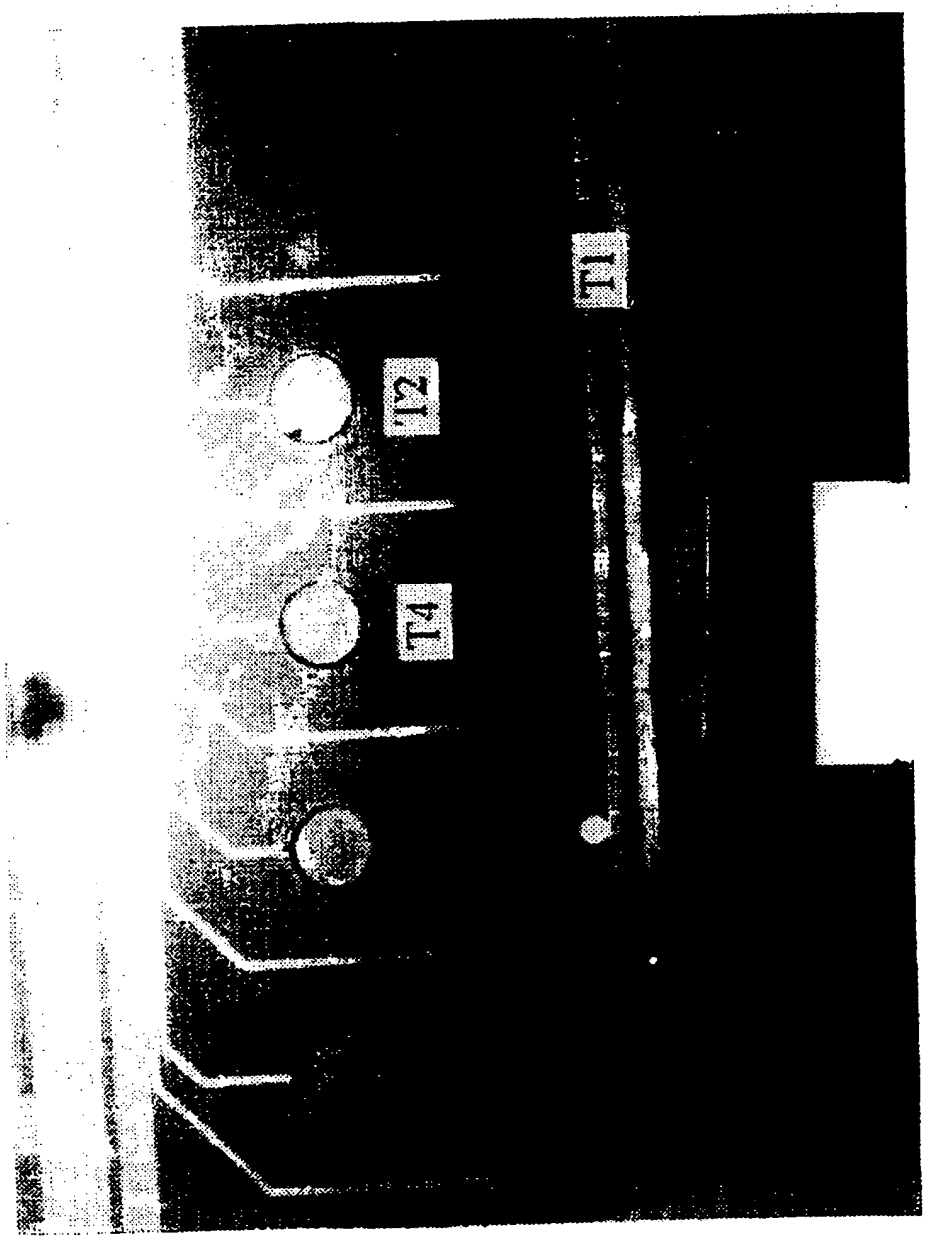

FIG. 23

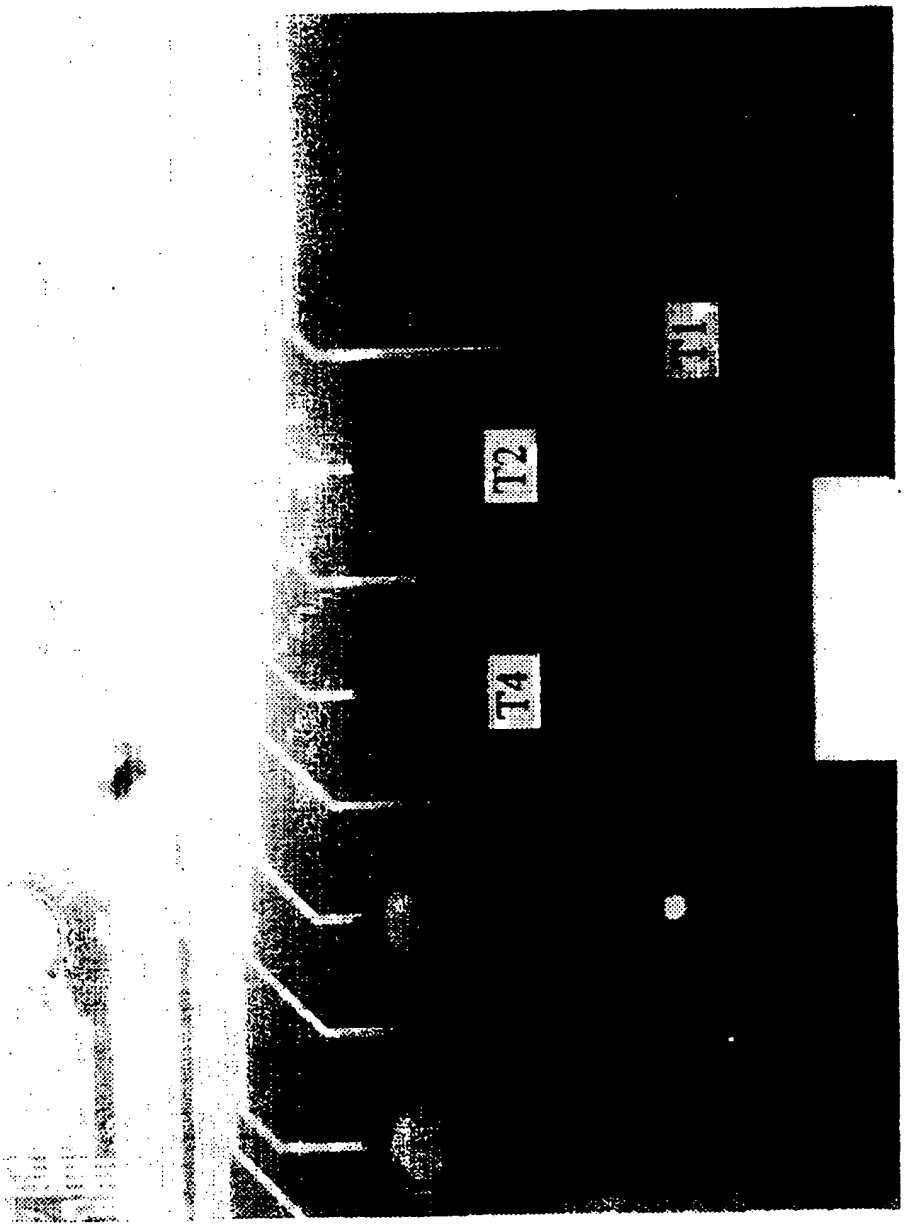

FIG. 24

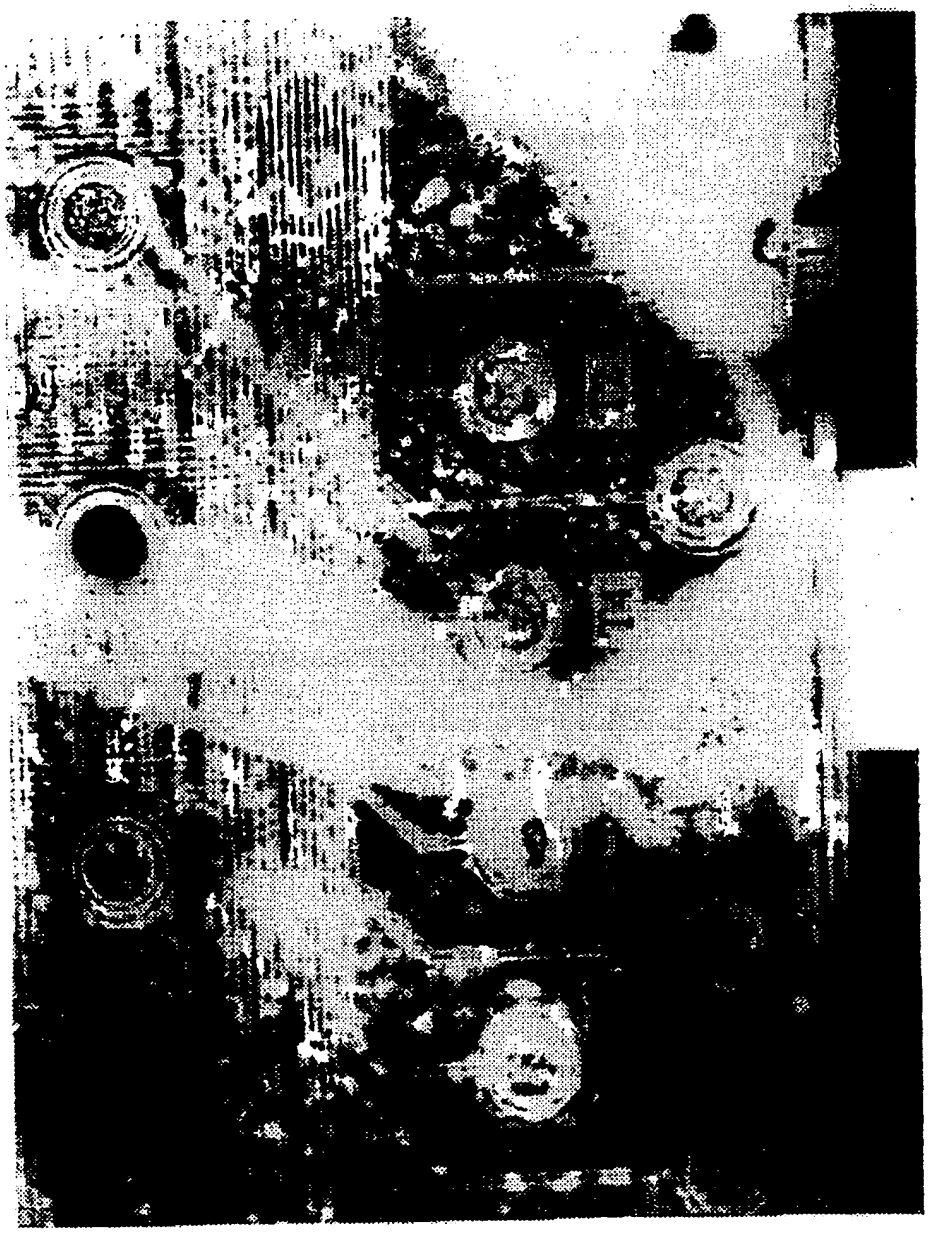

FIG. 25a

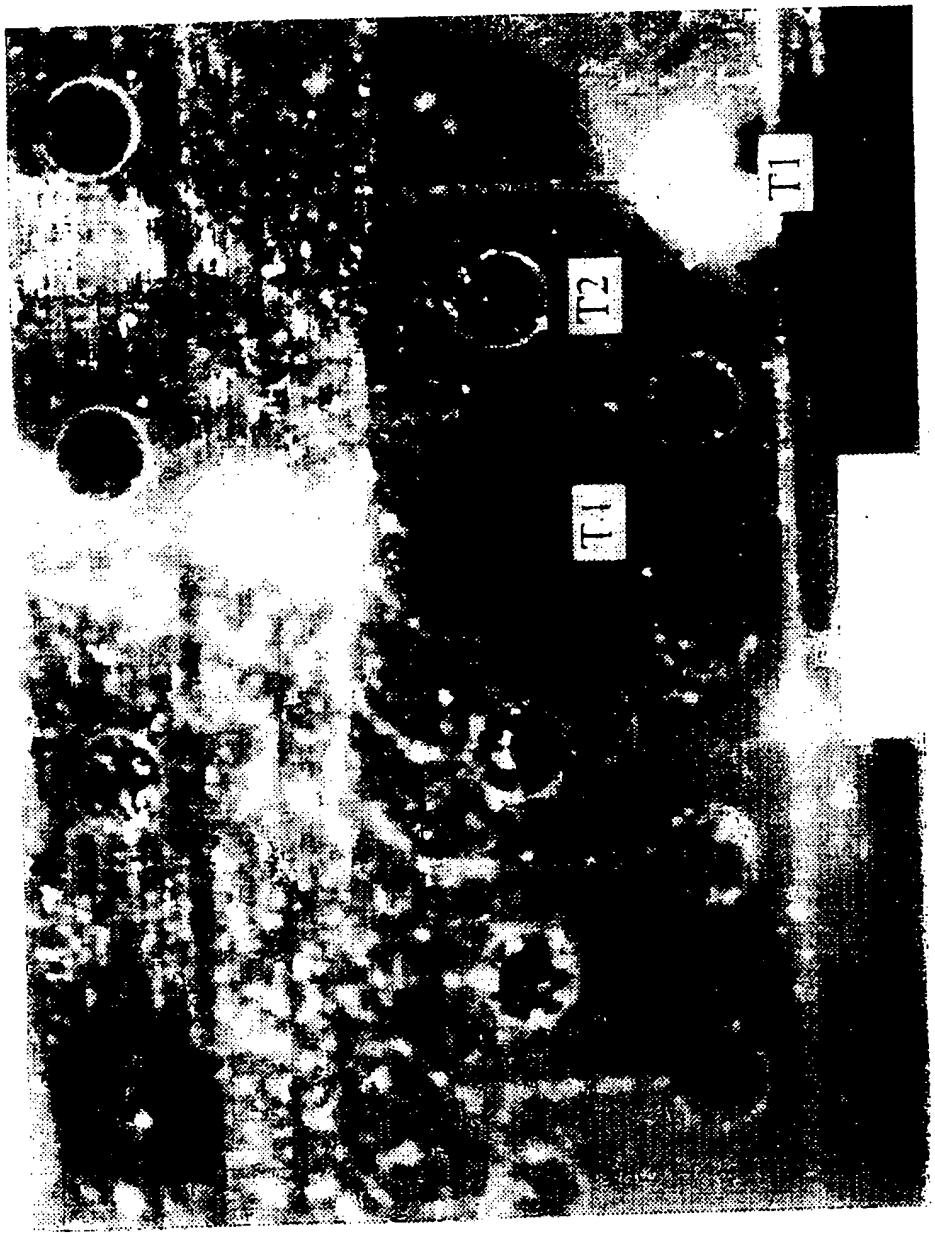

FIG. 25b

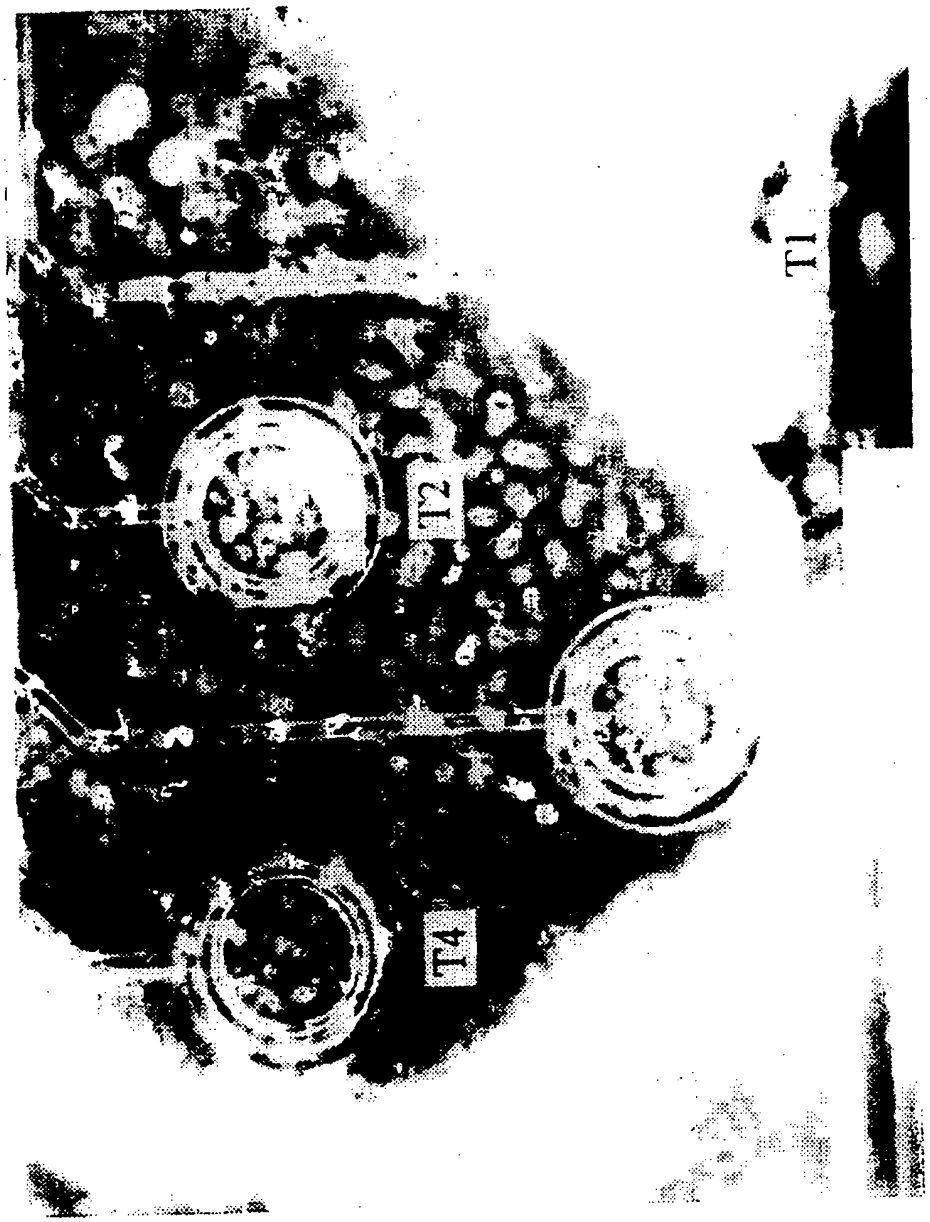

FIG. 26a

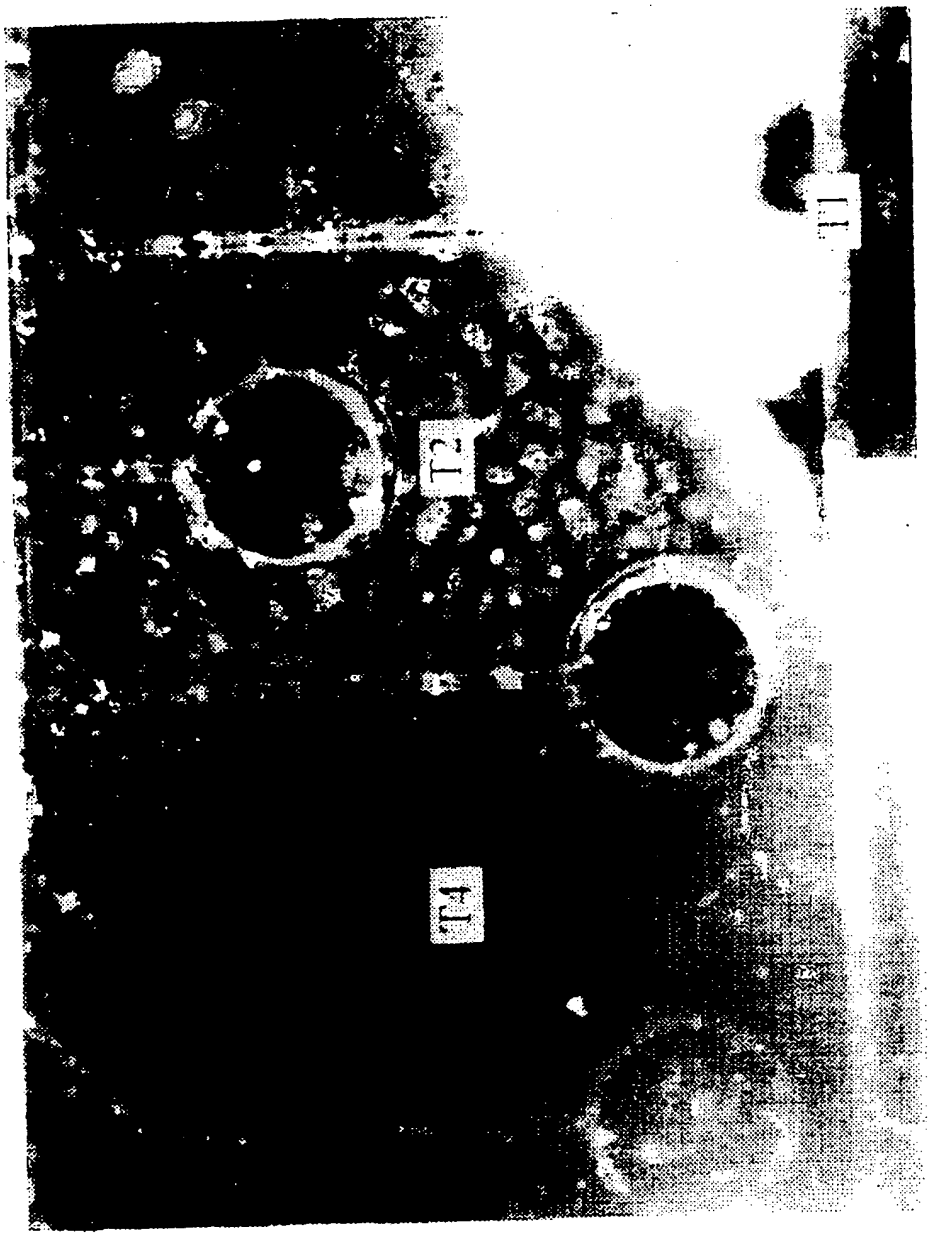

FIG. 26b

INTERNATIONAL SEARCH REPORT

Internat...al Application No
PCT/US 97/11463

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 B01J19/00 C07H21/00 C07K1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01J C07H C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 703 359 A (CIS BIO INTERNATIONAL (SOCIÉTÉ ANONYME)) 7 October 1994 see abstract see page 2, line 27 - line 33 see page 6, line 15 - line 22 see page 8, line 19 - page 9, line 7 see page 9, line 30 - page 10, line 31 see page 11, line 29 - line 36 see page 12, line 19 - page 13, line 11 see claims 13-18; examples 4,5	1,8
A		6,9-11, 13-17, 23,24, 26,28, 33, 35-38, 41,42,44

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1997	06.11.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stevnsborg, N

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/11463

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 07917 A (NANOGEN) 14 March 1996 see abstract; claims; figures -----	13,14, 28-32, 39,40

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/11463

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2703359 A	07-10-94	AT 159028 T	15-10-97
		EP 0691978 A	17-01-96
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WO 9607917 A	14-03-96	US 5632957 A	27-05-97
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		FI 970957 A	07-05-97

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C01B 3/58, H01L 23/26, C08K 3/22 — A1

(11) International Publication Number: **WO 97/11905**

(43) International Publication Date: 3 April 1997 (03.04.97)

(21) International Application Number: PCT/US96/15540

(22) International Filing Date: 27 September 1996 (27.09.96)

(30) Priority Data:

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(57) Abstract

The present invention is a hydrogen getter and method for formulating and using the getter. This getter effectively removes hydrogen gas typically present in many hermetically-sealed electronic applications where the presence of such gas would otherwise be harmful to the electronics. The getter is a non-organic composition, usable in a wide range of temperatures as compared to organic getters. Moreover, the getter is formulated to be used without the need for the presence of oxygen. The getter is comprised of effective amounts of an oxide of a platinum group metal, a desiccant, and a gas permeable binder.

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HYDROGEN AND MOISTURE GETTER AND ABSORBER FOR SEALED DEVICES

This application is entitled to priority pursuant to provisional Application Serial No. 60/004,449 filed September 28, 1995, entitled Hydrogen Getter. The U. S. Government has rights in this invention pursuant to contract number DE-AC04-76-DP00613 with the United States Department of Energy.

Background of the Invention

This invention generally relates to hermetically-sealed electronic devices and, more particularly, to compositions and methods for removing hydrogen and moisture from such devices.

Electronic assemblies for use in a variety of harsh environments, such as in space or marine applications, oftentimes are sealed from the outside conditions by use of gas-tight (hermetically sealed) containers. Such containers, while sealing out external dust, air and the like, trap in whatever is inside the devices.

Such hermetically sealed electronic assemblies generally contain semiconductor devices made from gallium arsenide (chemical symbol: GaAs) and/or indium phosphide (InP), and, in order to assure reliability and minimize failures in use, are test-operated at elevated temperatures (typically in the range of 125°C) for a period of time (typically 1,000 hours), generally referred to as burn-in and life testing. At such conditions, a loss of performance was found both in alternating current (AC), including radio frequency (RF), operating characteristics, as well as direct current (DC) operating characteristics. It was determined that hydrogen gas was the primary cause of the performance degrading. This degradation was later observed to be common to most GaAs semiconductor devices utilizing the industry standard gate metallization structures. (Structures made with titanium/platinum/gold (Ti/Pt/Au) or titanium/palladium/gold (Ti/Pd/Au) gates)(Kayali, "Hydrogen Effects on GaAs Device Reliability," 1996). The source of the problem has been determined to be hydrogen gas that was absorbed in the package metals (Kovar, etc.) or hydrogen generated by other materials such as RF absorbers inside the device package. Hydrogen gas desorbed or generated inside hermetically sealed devices has no clear path for escape. and its concentration can easily rise to 1-2% of the gas volume in gas-filled devices during initial operation.

The exact mechanism by which hydrogen degrades device performance, and the path by which hydrogen reaches the active area of a device is not well understood and is still a subject of investigation. However, it appears that the platinum group metals Pt and Pd used in gate structures play an important role in the degradation process (Camp et. al., "Hydrogen Effects on Reliability of GaAs MMICs," GaAs IC Symposium, 1989). These metals are catalysts for the dissociation of molecular hydrogen to atomic hydrogen which can diffuse into other areas of the device. Earlier research on GaAs transistors identified the diffusion of atomic hydrogen directly into the channel area of the device where it neutralizes the silicon donors as a possible mechanism (Chevallier et.al., "Donor Neutralization in GaAs(Si) by Atomic Hydrogen," Appl. Phys. Lett. 47, pg 108, July 1985.) Whatever the precise mechanism of device degradation by hydrogen may be, it is clear that it has a direct impact on the performance and reliability of GaAs devices used for high reliability applications.

One typical means of addressing this problem is the use of compositions which scavenge the hydrogen, generally referred to in the art and herein as "hydrogen getters" or, simply, "getters." Microcircuit devices, and, thus, the getters may be required to function in a vacuum, or be air or inert gas filled devices. Furthermore, they typically are required to function at temperatures ranging from -55°C to 150°C, or higher. Space inside such devices is limited and, thus, it is highly preferable that the getter may be easily formed into a thin film or similar shape to conform to the inside of the sealed device. Further, to be a viable remedy, the getter should lower hydrogen concentrations to the low parts per million level (100 PPM or lower is preferable) over the operating temperature range of -55° to 150°C where GaAs or similar devices are commonly used. It also must not desorb any other materials that might degrade device performance. Ideally, it should be easily manufactured to any desired physical dimensions and have a hydrogen capacity which could be tailored for any particular application.

One type of prior art hydrogen getter consists of alloys of metals such as iron, nickel, titanium, vanadium, zirconium, chromium, cobalt, the rare earth metals, and other metals and alloys which react with hydrogen to form metallic hydrides. These metallic getters typically require high temperatures exceeding 300°C for activation and/or operation, and are frequently poisoned by the presence of oxygen, water vapor, or other

contaminants such as chlorine gas and the like. Thus, this type of hydrogen getter may be undesirable where power or temperature constraints limit the hydrogen getter temperature or such contaminants are present. As other metals, these alloys can be metal-worked to form thin films for insertion into sealed devices, although the metal-working machinery to perform such stamping may be unwieldy and shaping into complex forms may be difficult.

Another type of prior art hydrogen getter consists of a mixture of a platinum group metal, usually palladium for cost reasons, on a substrate, such as activated carbon, to increase active surface area and an unsaturated organic compound, such as diphenyl butadiyne or 1,4-bis(phenylethynyl) benzene. The platinum metal operates as a catalyst in the mixture to bind the hydrogen into the organics. This type of getter will function at low to moderate temperatures (less than 100°C) and is not poisoned by oxygen or water vapor. However, these materials have significantly high vapor pressures at temperatures above 100°C and may melt in that range, which may lead to material migration inside the device, which is very undesirable. Although this type of getter is not poisoned by the presence of water vapor, neither does it have any capabilities for absorption of water vapor. Any water vapor present or produced inside of an electronic enclosure is a potential source of corrosion, and circuit shorting and thus is undesirable. Typically, this type of getter is manufactured from a commercially-available palladium-on-activated-carbon powder which is mixed with the desired organic material in a jar mill or the like to form a finely-ground and well-mixed powder. The powder may then be molded into a pellet shape in a pellet maker. However, the resulting mixture is very brittle and, thus, is not suitable for molding into thin shapes.

Summary of the Invention

It is thus an object of this invention to provide a hydrogen getter which will function in a vacuum or, alternatively in the presence of an inert gas, oxygen, air, and/or water vapor, in a wide range of temperature, from about -55°C to 150°C without being harmed thereby, to lower hydrogen levels to the equivalent of lower parts-per-million levels in sealed containers so that the components contained therein may effectively be protected from hydrogen gas.

In light of use of this hydrogen getter in cavities and as linings to electronic equipment, it is a further object of this invention to provide a hydrogen getter which is easily moldable into a variety of shapes and can be pressed or molded into electronic enclosures as a lining. The getter should not migrate in the enclosure at operating temperatures or produce dusting or flaking or evolve organic vapors.

It is a further object of this invention to provide a hydrogen getter of the type described which also functions as a desiccant in order to scavenge excess water in the enclosure and/or water produced by reaction of the hydrogen with the getter.

Further objects and advantages of the present invention will become apparent from the specification and claims described herein.

The composition of the present invention to satisfy these objects of the invention is an oxidized platinum group metal serving as both catalyst and oxygen source to convert hydrogen to water, combined with an appropriate desiccant, and a binder for holding the components in a matrix. Thus, the composition of the present invention comprises effective amounts of palladium monoxide or other oxides of platinum group metals as both a catalyst getter and a reactant along with effective amounts of an appropriate desiccant and effective amounts of a binder and hardener. As used herein, "effective amounts" of palladium monoxide or other oxides of platinum group metals indicate the percentages or amounts of the oxide components to satisfactorily remove the hydrogen anticipated to be present in a particular environment. Similarly, as used herein, "effective amounts" of a desiccant indicate the percentages or amounts of the particular desiccant component used, to satisfactorily remove the water anticipated to be present in a particular environment. Further, as used herein, "effective amounts" of a binder and hardener indicate the percentages or amounts of binder effective to stabilize and make workable the combined ingredients, metal oxide, desiccant and binder. As used herein, "effective amounts" of a hardener mean sufficient hardener to stabilize the combined ingredients while minimizing outgassing of unwanted components or binding of reactive oxides.

Description of the Invention

In a vacuum or in an inert gas, no oxygen will be present to react with hydrogen. However, it is known that certain metal oxides, particularly those of the

platinum group metals (ruthenuim, rhodium, palladium, osmium, iridium, and platinum), as well as copper (II) oxide and silver (I) oxide are reduced to the metal by exposure to hydrogen gas (F. A. Cotton, G. Wilkinson, "Advanced Inorganic Chemistry," pg. 994, Interscience Publishers, 1972). Thus, oxides of platinum group metals act as both catalyst and reactant in the presence of hydrogen gas. An excellent example to this gettering reaction for palladium monoxide (PdO) is shown below:

$$PdO + H_2 \longrightarrow Pd + H_2O$$

It is noted that PdO is the preferred getter, primarily because of cost reasons. However, other oxides of platinum group metals such as platinum, rhodium, rhenium, osmium and iridium may also be used for this purpose. These reactions are essentially irreversible and proceed easily even at temperatures as low as -55°C and above 200°C. Ag(I) decomposes at 160°C, for that reason, is undesirable in the present invention. Cu(II) is anticipated to be sufficiently slow in reaction that it is considered undesirable in the present invention. However, if these constraints are acceptable in a particular application, oxides of Ag(I) and Cu(II) may be acceptable in a getter.

However, such oxides alone are not suitable for use as getters because, among other things, the reaction above produces water which would also present a problem to sealed electronic circuits. Addition of an appropriate desiccant medium to collect resulting water, such as a molecular sieve, removes the water produced. Molecular sieve desiccant is preferred because of its capacity for water, typically 22 percent by weight, and lack of swelling or dusting. However, the platinum group oxides and desiccant alone are not suitably moldable for use in sealed containers.

The combination of platinum group oxides and desiccant mixed into a gas permeable binder, in proper proportions forms a moldable material which will both react with hydrogen and capture the water produced. For example, a room temperature vulcanized (RTV) silicone is gas permeable and stable at temperatures below -55°C and above 200°C.

Combined in proper proportions, the platinum metal oxides, desiccant and binder will catalyze the reaction between hydrogen and oxygen and the desiccant sieve will adsorb the water produced by the reaction. This getter system requires no activation for the reaction of hydrogen with oxygen, but can be activated for water absorption by

conventional means such as baking at high temperatures (i.e., above 150°C), using vacuum, or inert gas purge. After such baking, the system should not only be dry, but will also not evolve organic vapors which might harm microcircuit components. The system will function in microcircuits even during the initial "burn-in" of the device at temperatures up to 150°C. In particular, a system of RTV silicone, molecular sieve desiccant and PdO will function up to at least 200°C without softening and will produce no volatile organic compounds that might damage sensitive components.

The described getters for both the air-filled devices and the evacuated/inert gas-filled devices are moldable materials that can be cast and cured in any desired shape and size, including thin films (less than 0.010 inches thick) for use in microcircuit devices where little space is available. They may also be cast in place in such a device, eliminating the need for any adhesive bonding.

Preferred Embodiment

The preferred embodiment of the composition of the present invention, for cost, availability and similar considerations, is palladium monoxide and a molecular sieve desiccant (zeolite) mixed in appropriate proportions and added as a homogeneously mixed filler to a curable silicone elastomer to form a matrix. This combination can be molded to any desired dimensions and the amount of PdO and desiccant may be adjusted to handle the anticipated amount of hydrogen and moisture for any particular application. The molecular sieve desiccant will scavenge the water produced by the reaction of the metal oxide with hydrogen. The silicone elastomer is an excellent binder for the oxide and desiccant, and has very high permeability to both hydrogen and water vapor. When properly postcured, the getter is stable up to 200°C and does not outgas any organic volatiles that might prove detrimental to a sealed device.

Given the nature of the present invention, it is intuitive that there is no theoretical lower limit to the amount of PdO that could be used in the present invention. Rather, changing the percentage amounts of PdO simply changes the rate at which hydrogen is absorbed and the total amounts of hydrogen absorption which can occur. The total theoretical capacity of the present invention for hydrogen is a simple chemical balance of the available moles of oxygen contained in the PdO, reacted with the hydrogen. For practical reasons, the amount of active ingredients, PdO and desiccant will

typically be maximized, to minimize the total amount of present invention needed to remove the hydrogen anticipated. However, as discussed below, high proportions of solids, the PdO or other platinum group oxides and the desiccant, will make the resulting mix unacceptably stiff and hard to mold. Thus, if typically between about 10 percent and about 30 percent by weight of the total composition of the present invention getter is platinum group oxides, the getter will be sufficiently reactive, and should still be acceptably moldable.

It was discovered that, although various forms of palladium monoxide function in the present invention, the gettering capacities of palladium monoxide formed by particular processes is preferred. It appears that palladium monoxide formed as a result of a process by which palladium is dissolved in a strong acid, such as aqua regia, and refined by forming salts thereof in a refining process to ultimately form a refined palladium monoxide powder is significantly more active than palladium monoxide formed by decomposition of palladium nitrate ($Pd(NO_3)_2$). The gettering capacities of palladium monoxide formed by the former process are roughly two to three times as effective at removing hydrogen than palladium monoxide formed by the latter process. Thus, depending upon cost, availability, and space considerations, use of the palladium monoxide formed by the salt refining technique is generally preferred to palladium monoxide formed by decomposition of nitrates. Thus, it will be apparent that effective amounts of palladium monoxide will vary, in part, depending upon the process used to produce the palladium monoxide.

The preferred desiccant for the present invention is a molecular sieve desiccant, generally for capacity and size stability reasons. Molecular sieve desiccant is available in several size ranges from 3 Å to 15 Å, any of which is expected to work effectively. However, the 3 Å is generally the most preferred size because it characteristically will absorb few types of molecules except for water. The proportion of desiccant used is related to the amount of water expected to be produced. For example in a vacuum application with only hydrogen expected. the only water produced would be from reaction with the platinum group oxides. A 3 Å sieve has a nominal 22 percent by weight water capacity. For PdO, with a molecular weight of 122, water with molecular weight of 18 will be produced which is equivalent to 15 percent by weight of the original

PdO. Thus, for each unit weight of PdO, 15/22 or .68 units weight of 3 Å is required. For most applications, excess desiccant is desirable, however, to avoid potential free water in the enclosure. Thus, the portion of desiccant required will typically be from about 5 to about 35 percent by weight of the total mixture.

As noted in the following examples, workability of the preferred embodiment of the present invention prior to curing decreased significantly at about 45 percent by weight of the RTV silicone used - type GE 615 as manufactured by General Electric Company of Waterford, New York, (GE 615). Thus, the practical limits for binder appear to be from about 85 percent to about 35 percent by weight of the total composition of the present invention, including platinum group metal oxide, desiccant, and binder.

As discussed below in Example 15, the amount of hardener used in the binder can have a significant impact upon the effectiveness of the getter. Basically, use of the manufacturer's recommended amount of hardener when used with the RTV silicone binder severely inhibits the ability of the hydrogen getter to operate. It appears that the amounts of binder typically used in the RTV silicone to assure a complete binding reaction in all parts of the temperature range and for various mixing techniques produce excess amounts of hardener in the final product. This excess hardener may be a hydrogen generator and, thus, the excess hardener has a direct impact upon the effectiveness of the present invention. As indicated in the cited example, basically the minimum amount of hardener to form a stable binder should be utilized to maximize the gettering capability of the resulting composition of the present invention. The preferred mix of ingredients using the GE 615 RTV silicone was about 95 parts resin to about 5 parts hardener.

In mixing the composition of the present invention, especially in light of the need to minimize the amount of hardener utilized in any binder, the gettering material and desiccant should preferably be mixed prior to introduction into the binder. The binder resin should be thoroughly mixed with the setting agent or hardener prior to introduction of the amounts of desiccant and getter material. In this fashion the hardener will be as thoroughly mixed with the binder as possible prior to introduction of the getter material so as to minimize the contact of the getter material with free unreacted hardener

components. The amounts used should be amounts effective to satisfactorily perform the hydrogen gettering desired. Upon mixing of the components, the mixture should be promptly shaped or molded into the desired shape prior to the hardener acting. As noted in the following examples, the mix is then preferably cured by heating, followed by activation of the desiccant.

It is noted that, although oxides of the palladium group metals, including PdO, will catalyze hydrogen and oxygen directly, the catalytic reaction proceeds more slowly than an equivalent reaction with a nonoxidized palladium group metal. For this reason, small amounts of a palladium group metal may be added to the present invention in instances when oxygen is expected to be present and a faster reaction is desired. Typically, this will be a commercially available palladium on a substrate such as activated carbon. It is also noted that various other additives could be utilized along with the present invention to perform specific functions in particular situations. For example, as noted herein, the present invention works effectively in the presence of RF absorbers. Thus, in particular instances, the present invention could be combined with an RF absorber substance compatible with the binder to form a combination RF absorber and hydrogen getter. One advantage of such a combination would be additional space savings in the sealed enclosures.

Various embodiments of the current invention are further illustrated by the following nonlimiting examples:

Example 1

A formulation of the present invention using PdO was prepared with 15% by weight PdO, 45% by weight 3Å molecular Sieve (3Å), and 40% by weight GE 615. Except as noted, PdO used in all examples is salt deposited PdO, Catalog No. 99210, manufactured by Alfa Aesar; a Johnson Matthey Co., 30 Bond St., Ward Hill, Mass., 01835-0747.

A 20-gram sample of the above formulation was hand-mixed. The material was very viscous at this level of solids loading (60%), so mixing was difficult, and the mixture would not flow easily. The mix was pressed to a 0.010 inch thick sheet and cured at 75°C for about 4 hours. It was then placed into the vacuum oven at 185°C and below 0.1 mm Hg pressure for final cure and drying of the molecular sieve powder.

Sample size was 1.5 grams, and the amount of hydrogen injected was 0.75 cc. A 150 cc volume test chamber was used. Results are shown in Table 1:

Table 1

Time	%H_2*	Pressure, torr	Action
8:10:00 am		761	Initial nitrogen pressure
8:18:30		765	Added 0.75 cc hydrogen
8:20:30	0.7574	761	Removed 0.5 cc gas sample
8:26:30		759	Pressure reading
8:47:30		758.5	Pressure reading
8:48:30	0.0192	756	Removed 0.5 cc gas sample
1:03:30 pm		756	Pressure reading
1:03:45	0.0020	753	Removed 0.5 cc gas sample

*Unless otherwise noted, all % and ppm gas readings herein are per volume.

By the end of the test period, the hydrogen concentration had dropped to approximately 20 ppm, which is near the detection limit for the hot wire detector used in the Perkin-Elmer Company of Norwalk, CT gas chromatograph (Model Sigma 2000 GC) utilized for the sample analysis shown in Table 1.

On day 2 of this test at 8:20:15 am, pressure was 752 torr. At 8:21:00 am, 0.0011% H_2 was detected upon removal of 0.5 cc gas sample at pressure of 750 torr.

On day 3 of this test, H_2 detection was switched to a Model 3202 Trace Gas Analyzer made by Valco Instruments Co, Inc, of Houston Texas (VICI GC) for more sensitivity. On this day, results at 11:31 am showed 0.739 ppm hydrogen and 743 torr pressure upon removal of a 1.0 cc gas sample.

On day 9 of this test, results using the V1C1 GC were at 9:35 am, 0.070 ppm hydrogen detected at 739 torr upon removal of a 1.0 cc gas sample. This test showed significant continued long-term scavenging of H_2 by the present invention getter.

Example 2

In order to test acceptable mix viscosity. two more formulations of getter were made. The first new formulation was 15% by weight PdO. 40% by weight 3Å Mol.

Sieve, 45% by weight GE 615 Silicone. A 20 gram sample of this formulation was hand-mixed and pressed into a 0.010 inch thick sheet. It was observed that this mixture was still very viscous, and would not flow easily.

The second new formulation was 15% by weight PdO, 30% by weight 3Å Mol. Sieve, 55% by weight GE 615 Silicone. A 20-gram sample of this composition was hand-mixed and pressed into a 0.010 inch thick sheet. This mix was less viscous and would flow, although slowly.

Both mixes were cured for 4 hours at 75°C and then put in the vacuum oven at 185°C and less than 0.1 mm Hg pressure for final cure and desiccant drying. Both mixes cured properly and performed satisfactorily as getters. However, it appears that for minimal molding characteristics, the amount of binder, such as GE 615, in the present invention should be maintained above about 35% by weight. Preferably, higher levels of binder, about 55-65 by weight, increase material moldability. If molding characteristics are not a significant concern, further reductions in the amount of binder may be acceptable.

Example 3

Further testing on the efficacy of the present invention proceeded by preparation of a 1.38 gram of a test of formulation of 15% by weight PdO, 30% by weight 3Å, and 55% by weight GE 615, prepared as noted in Example 2. This sample was put in a 150 cc volume test chamber, which was evacuated and then backfilled with nitrogen gas and tested with the addition of 0.75 cc hydrogen gas added through a septum. The resulting data is shown in Table 2 below:

Table 2

Time	$\%H_2$	Pressure, torr	Action
10:13:45 am		770	Initial nitrogen pressure
10:25:45		774	Added 0.75 cc hydrogen gas
10:27:45	0.5153	770	Removed 0.5 cc gas sample
10:41:30		767	Read pressure only
10:55:45	0.0137	764	Removed 0.5 cc gas sample
2:25:45 pm	0.0016	762	Removed 0.5 cc gas sample
2:40:45	0.377 ppm*	757	Removed 1.0 cc gas sample
11:57:00 am (next day)		757	Pressure Reading only
11:58:10 am		753	Removed 1.0 cc gas sample, no reading, GC malfunction
12:47:00 pm	Non-detect	748	Removed 1.0 cc gas sample. (ppm H_2 below peak threshold (PT))
1:08:00 pm	0.091 ppm	743	Removed 1.0 gas sample, dropped Peak Threshold (PT) level to .015 ppm

*Switched to the VICI GC for greater sensitivity; results for hydrogen are in ppm rather than percent as above.

Example 4

One problem with hydrogen getters involved producing getters which are sufficiently thin for use in small component applications. This example documents an attempt to make 0.005 inch thick sheet sample.

A 20 gram sample of a 15% PdO, 30% 3Å, 55% GE 615 formulation was hand-mixed and pressed to a thickness of 0.005 inches using steel plates separated by 0.005" shim stock. The samples were not very uniform, and many holes appeared. They seem to be due to surface tension of the mix. Three samples were cast and placed in a 75°C oven for 3 hours for the initial cure. When removed from the oven, the samples were found not to be fully cured, and were very difficult to remove from the Teflon cloth because they tore very easily. Two of the samples were

removed and put in the vacuum oven at 185°C and less than 0.1 mm Hg pressure for final cure and drying. The third sample was left in the 75°C oven overnight.

The sample cured at 75°C overnight was improved in cure state. It was placed in the 185°C oven for final cure. The other two samples that had been cured overnight at 185°C in vacuum were placed in sample jars and put into the glove box pending reaction testing. These samples appeared to be fully cured indicating that the present invention satisfies the need to reliably produce thin getters.

Example 5

One primary use for hydrogen getters is for use with radio frequency (RF) absorbers. This is because RF absorber material typically is a major source of hydrogen outgassing into electronic enclosures. The RF absorber is necessary in these enclosures to eliminate spurious radio frequency signals which could disrupt the proper operation of the electronics in the enclosure. Thus, it is important for hydrogen getters to operate effectively in the presence of RF absorber materials.

In order to test effectiveness of the present invention in the presence of RF absorbers, a 2-inch (5.1 cm.) by 3-inch (7.6 cm.) strip .010-inch (.025 cm.) thick sample of getter material 15% by weight PDO, 25% by weight 3Å, 60% by weight GE 615, was hand-mixed and pressed to the indicated thickness, cured, and cut to size. A 3-inch (7.6 cm.) by 4-inch (2.2 cm.) strip which was .020 inch (.051 cm.) thick of RF absorber material was loaded into 110 cc stainless steel vessels. The hydrogen content was analyzed by gas chromatography at various intervals. The vessels were filled with dry nitrogen at 1100 torr and maintained at 150°C during the test. Two industry standard RF absorbers were tested, both metal impregnated silicone binder absorbers. Two absorber control vessels were used with each absorber formulation, as well as an empty vessel for reference of hydrogen desorption for metals. Also, a sample of each absorber with the indicated amount of getter was produced. The results were as shown in Table 3 below:

Table 3

Hydrogen Concentration (PPM)

Elapsed Time (Hours)	Absorber Formulation A	Absorber Formulation A	Absorber Formulation A with Getter	Absorber Formulation B	Absorber Formulation B	Absorber Formulation B with Getter	Empty Vessel (control)*
0	0	0	0	0	0	0.000	0.0
140	1660	1350	0.060	344	361	0.073	12.70
237	2531	1971	0.027	716	680	0.052	25.39
408	3312	2653	0.057	1168	1091	0.030	36.53
672	4397	3578	0.020	2017	2009	0.020	no data
1008	5215	5140	0.065	3424	3131	0.047	408

* Control simulates hydrogen desorption from metals.

Based upon these results, the present invention functions well to remove unwanted hydrogen in the presence of RF absorbers.

Example 6

A moisture absorption test was performed utilizing a getter sample prepared the same as the getter sample used in Example 5 above (a 15% PdO, a 25% 3Å, 60% GE 615) formed into a 2 inch x 2 inch (5.1 cm x 5.1 cm.) strip nominally 0.020 inch. (.051 cm) thick. These samples were activated in an evacuated oven at 150°C for 16 hours. The test samples were then inserted into a humidity chamber maintained at 35% relative humidity (within +2 to -15% relative humidity), and 75°F plus or minus 5°F (24°C plus or minus 3°C). The results are shown as percent weight gain in the samples and are summarized below in Table 4:

Table 4

Test Part Weight, grams

Elapsed Time (Hours)	Sample #1	Sample #2	Sample #3	Sample #4
0	2.69	2.90	2.77	3.14
1	2.74	2.95	2.82	3.19
2	2.77	2.97	2.85	3.21
3	2.79	3.00	2.86	3.23
5	2.81	3.02	no data	no data
20	no data	no data	2.92	3.32
21	2.84	3.05	test complete	test complete
23	2.85	3.05	test complete	test complete
24	test complete	test complete	test complete	test complete
Total Gain	0.16	0.15	0.15	0.18
Percent Gain	5.9%	5.2%	5.4%	5.7%

Theoretical weight gain for water absorption is 5.5% by weight based upon a nominal capacity of the 3Å molecular sieve of 22 percent by weight. The test results confirm the theoretical desiccating capacity of the present invention.

Example 7

Tests were run on hydrogen absorption on aged getter material of the present invention mixture compounded as described in Example 5 above (a 15% PdO, 25% 3Å molecular sieve, 60% GE 615). The material tested was a 1.5 inch x 2 inch strip (3.8 cm. x 5.1 cm) nominally 0.020 inch (.051 cm) thick. The samples were aged 984 hours at the temperatures noted in Table 5 inside sealed stainless steel vessels. Samples were then placed in 150 cc glass vessels and tested at room temperature ("RT," nominally 71°F/22°C). A 2.5 cc portion of hydrogen was injected into each sample vessel at time = 0. The results are summarized in Table 5:

Table 5

Hydrogen Concentration (PPM)

Elapsed Time (minutes)	RT	60°C	95°C	125°	Gas Chromatograph
0	16667	16667	16667	16667	Initial calculated concentration
60	2402	2267	1950	2416	Perkin-Elmer 0.5 cc injection
120	490	464	505	519	Perkin-Elmer 0.5 cc injection
180	172	123	161	127	Perkin-Elmer 0.5 cc injection
240	37	94	38	21	Perkin-Elmer 0.5 cc injection
300	0.962	0.591	0.206	0.406	VICI-GC 5.0 cc injection

Results indicate no loss of gettering capacity with aged material of the present invention.

Example 8

Example 8 is a compilation of hydrogen absorption rate testing performed at room temperature on a sample of the material of the present invention as formulated in Example 5 (15% PdO, 25% 3Å molecular sieve, 60% by weight GE 615) cut into 1 inch x 4 inch strips (2.5 cm x 10.2 cm), .010 inches (.025 cm) thick. Samples were inserted to 150 cc volume stainless steel vessels filled with dried nitrogen. Two cc of hydrogen were added at time = 0. Results are summarized in Table 6 below:

Table 6

Hydrogen Concentration (PPM)

Elapsed Time (minutes)	Sample #1	Sample #2	Gas Chromatograph
0	13333	13333	Initial calculated concentration
2	12873	12989	Perkin-Elmer 0.5 cc injection
15	1813	2222	Perkin-Elmer 0.5 cc injection
30	320	409	Perkin-Elmer 0.5 cc injection
60	44	74	Perkin-Elmer 0.5 cc injection
120	1.878	2.500	VICI-GC 5.0 cc injection
240	0.057	0.089	VICI-GC 5.0 cc injection

Results indicate a high rate of absorption, a reduction in more than 99 percent of available hydrogen in 1 hour.

Example 9

Example 9 is a set of kinetics data for a certified hydrogen leak rate. Material tested is a mixture as shown in Example 5 (15% PdO, 25% 3Å molecular sieve, 60% GE 615) cut into a 1 inch x 6.2 inch strip (2.5 cm x 15.7 cm), .010 inches (.025% cm) thick glued to an equal size sheet of aluminum foil. The material was inserted into 150 cc glass test vessels with a 4×10^{-7} cc (STP)/sec certified hydrogen leak. The vessels were nitrogen filled at 800 torr. Gas samples were removed at times noted and analyzed for hydrogen concentration by gas chromatography. The results are summarized in Table 7:

Table 7

Hydrogen Concentration (PPM)

Elapsed Time (Hours)	Room Temperature	Elapsed Time (Hours)	60°C	Elapsed Time (Hours)	-10°C	Elapsed Time (Hours)	95°C
0	0.000	261.0	5.041	330	14.320	596.67	2.576
115.5	7.030	288.3	4.808	357.5	13.469	610.2	2.118
140.6	7.775	309.0	5.590	480	17.260	681.3	2.390
162.5	7.840	980.6	5.195	499.2	17.250		
		1008.2	5.762				
Average	7.548	Average	5.339	Average	15.993	Average	2.254

Results show a long-term stability of the present invention gettering rate for a set leakage rate and, also, a direct dependance of gettering rate versus temperature. This also indicates effective gettering of the present invention over a wide range of temperatures, beyond the temperatures tested.

Example 10

In order to confirm that the theoretical capacity of the getter was consistent at varying levels of PdO, a sample of 25% PdO, 20% 3Å molecular sieve, 55% GE 615 was prepared. At this ratio, the theoretical capacity of the mixture calculated as described herein. is 45.75 cc atm/gm. (Herein, "atm" indicates at standard temperature and pressure - STP.) Experimental value derived from data was 44.6 cc atm/gm. This close correlation

indicates that the actual hydrogen capacity of the present invention should be very close to the theoretical capacity over generally any ratio of component mixtures.

Examples 11, 12 and 13

Examples 11, 12 and 13 were performed utilizing 5% by weight PdO, 5% by weight Pd on C, 30% by weight 3Å molecular sieve, and 60% by weight GE 615 mixed as shown below.

First, the PdO and Pd on carbon were combined mechanically by placing 5.1467 grams of PdO and 5.1540 grams of 5% Pd on carbon catalyst in a small ceramic ball mill jar with ceramic grinding cylinders and ball-milled overnight. This produced a finely divided uniform mixture of the two materials, PdO and 5% Pd on carbon.

Next, 40 gram sample was hand mixed using 4.0 grams of the mixed PdO/5% Pd on carbon mixture, 12.0 grams of 3Å molecular sieve powder, and 24 grams of the premixed GE 615 RTV silicone with hardener.

After hand-mixing, the formulation was pressed into two approximately 16 gram sheets about 0.010 inches thick using 0.010 shim stock and Teflon cloth. The sheets were cured at 75°C for 4 hours. The sheet samples were then removed from the Teflon cloth, and placed in a vacuum oven at 185°C and less than 0.1 mm Hg pressure for final cure and drying. The samples were cured overnight in the vacuum oven.

Tests in Examples 11-13 were performed at room temperature, approximately 70°F (21°C).

Example 11

A sample of about 8 grams of this PdO/Pd test formulation (5% PdO, 5% Pd on C, 30% 3Å and 60% GE 615) was placed in the 150 cc test vessel. The air was evacuated and the volume backfilled with nitrogen (N_2). A 15 cc sample of H_2 gas was injected into the volume through a septum, and the pressure observed versus time. The results are shown below in Table 8:

Table 8

Time	%H_2	Pressure, torr	Actions
8:20 am		724	Starting N_2 pressure
8:21:30		796	Added 15 cc hydrogen gas
8:23:30		761	Pressure reading only
8:29:30		745	Pressure reading only
8:36:30		734	Pressure reading only
8:51:30		730	Pressure reading only
8:51:40	0.896	728	Removed 0.5 cc for analysis
9:02:00		727	End test #1

Example 12

The 150 cc test vessel was evacuated and refilled with clean nitrogen (N_2) for a second test, using same sample as test #1. Results are shown in Table 9:

Table 9

Time	%H_2	Pressure, torr	Actions
9:04:40 am		753	Refilled with nitrogen
9:05:40	0.1449	751	Removed 0.5 cc gas sample
9:15:00	0.2025	748	Removed 0.5 cc gas sample
9:26:30		747	Starting N_2 pressure
9:27:00		820	Added 15 cc hydrogen gas
9:29:00		795	Pressure reading only
9:34:00		778	Pressure reading only
9:42:00		768	Pressure reading only
9:57:00		760	Pressure reading only
9:57:15	1.846	758	Removed 0.5cc for analysis
1:26:30 pm		750	Pressure reading only
1:26:45	1.0393	748	Removed 0.5 cc for analysis

The PdO Test #2, was allowed to sit two days, and two more gas samples were taken on the third day as shown below in Table 10:

Table 10

Time	$\%H_2$	Pressure, torr	Action
8:27:00		741	Pressure reading
8:27:15	0.8199	739	Removed 0.5 cc for analysis
8:33:50	0.8212	737	Removed 0.5 cc for analysis

The hydrogen concentration appeared stable at approximately 0.8 percent. The 30 cc hydrogen added to this sample has used about 41 percent of the hydrogen gettering capability of this sample. The gettering rate appears to be slow at this level of loading.

Example 13

In this test, we used amounts of PdO test formulation and H_2 that more clearly represents the expected level of loading that might be seen in a microcircuit. We used a 1.6 gram sample of the test formulation (5% PdO, 5% Pd on C, 30% 3Å and 60% GE 615) in a 150 cc test vessel with a smaller .75 cc hydrogen loading. Results are as shown in Table 11 below:

Table 11

Time	%H_2	Pressure, torr	Action
9:00 am		760	Initial N_2 pressure
9:10		757	Wait for stable pressure
9:10:15	ND	755	Removed 0.5 cc for analysis
9:20:15		759	Added 0.75 cc hydrogen gas
9:22:15	0.7497	755	Removed 0.5 cc gas sample
9:49:15		751.5	Pressure reading only
9:50:15	0.0124	749	Removed 0.5 cc gas sample
1:50:30 pm	0.0023	746	Removed 0.5 cc gas sample
03:30	0.0015	744	Removed 0.5 cc gas sample
8:03:30 am (next day)	0.0015	744	Removed 0.5 cc gas sample

This test demonstrates very effective removal (gettering) of hydrogen with the PdO/Pd combined. The test vessel reached approximately 23 ppm hydrogen, approximately .3 percent of the initial level, in about 4.5 hours. The detection limit of the gas chromatograph (GC) being used was 15 ppm.

Example 14

Purity of the components of this hydrogen getter is of great importance. Of particular interest is the level of residual contaminates that remain on the palladium monoxide as purchased from a chemical supplier. Early aging tests performed with palladium monoxide purchased by catalog number 11040 from Alfa Aesar - Johnson Mathey identified a direct link to the amount of observed methane generated to the level of chlorine contaminate on the palladium monoxide. Ion chromatography on the PdO identified a chloride ion value well in excess of 1000 parts per million. Specific requests were made to several vendors to have the chloride ion count eliminated or reduced to maximum levels no greater than 200 parts per million.

Of concern to manufacturers of hermetically sealed electronic containing devices such as hybrid microcircuits is the identification of outgassing components from polymeric materials. Tests for outgassing are defined in Mil-Std-883, Method 5011.4. Gas

analysis of the internal volume of present invention getter samples undergoing accelerated aging was performed to identify, if any, gas species generated from the prepared getter material. This analysis performed by gas injection into calibrated gas chromatographs identified a significant level of methane gas. The conclusion drawn from this analysis was that there were impurities in the mix components of the getter.

Alfa Aesar successfully met the 200 parts per million requirement with two different preparations of palladium monoxide. Sample A (new catalog stock number 99210) met the 200 parts per million maximum level and tested to a consistent hydrogen capacity of 27 cc-atm per gram hydrogen in the prepared getter, in close agreement with the theoretical capacity of 27.4 cc-atm per gram. Repeated accelerated aging samples confirmed the removal of methane generated gas. Tests performed on other catalog number samples from various manufacturers were not as successful. The hydrogen capacity test repeatedly tested to between 10.5 and 13.4 cc-atm per gram. The accelerated aging samples however, showed no methane generation. An investigation of the total ionic contaminates using ion chromatography between Alfa Aesar sample "A" and the lower performing samples ("B") from various vendors identified a nitrate ion (NO_3^+) was present in large amounts (greater than 1000 ppm) in sample "B" but was not detected in sample "A."

Based on the above analysis we have concluded that the removal of chloride ions from palladium monoxide is required for elimination of methane gas from the prepared getter. The method of palladium monoxide preparation which removes or reduces these chlorine levels must not use a preparation process that leaves nitrate ions present. Subsequent attempts at removing the nitrate, washing, leaching, etc., from the nitrated contaminated palladium monoxide (the "B" samples)were made. Although the nitrate contamination was successfully removed, such removal had no apparent effect upon the gettering capacity of the "B"samples.

The chloride ion concentration present in the samples of Catalog No. 99210 from Alfa Aesar is characteristic of palladium monoxide prepared by a salt precipitation technique which makes use of an acid dissolved palladium and then repetitive salt precipitations to isolate the palladium monoxide thus produced. The amount of residual chloride ion is dependent upon the amount of refining performed to remove these

contaminants from the palladium monoxide produced. Production of palladium monoxide by this method is well known to one experienced in the art. The refining technique would not contaminate the samples with nitrates, however.

In comparison, palladium monoxide prepared by decomposition of palladium nitrate ($Pd(NO_3)_2$) would leave trace amounts of the nitrate in the sample. Thus, it was determined that the other commercial samples of palladium monoxide were produced by this second technique.

Poor performance of the second method of production of the palladium monoxide is opined to result from the difference in the crystalline structure of the palladium monoxide and binding of the molecules in the structure. As indicated, the palladium monoxide produced by the second technique is usable as a hydrogen getter, albeit at a lower activity level.

Example 15

In initial testing of the hydrogen capacity of the present invention getter, a considerable variation in the capacity value of the getter was observed, with all samples being significantly lower than the theoretical capacity. A getter containing 15% by weight PdO has a theoretical hydrogen capacity of 27.45 cc-atm per gram, based upon each mole of PdO reacting with a mole of hydrogen gas. The hydrogen capacity test is performed by exposing a sample of the present invention getter to an excess of hydrogen in a known calibrated volume and observing the change in pressure as the reaction proceeds. Capacity values were observed ranging from about 22 cc-atm/gram to as low as 15 cc-atm/gram. Upon investigation, it was discovered that most room temperature vulcanizing vinyl addition cure silicones, such as GE 615 RTV, are normally mixed with a large excess (20-50%) of silane (-Si-H) group-containing curing agent to hasten cure at room temperature. These excess silane groups in the cured material are a significant source of hydrogen evolution, as they react with water or other hydroxy group containing materials. See W. E. Dennis, D. A. Sierawski & D. N. Ingebrigston, "Hydrogen Evolving Tendencies of Cable Fillers and Optical Fiber Coatings," Rubber World, Volume 193, No. 2, November 1985, pp 26-29. We concluded that these excess silane groups were generating hydrogen which reacted with the PdO during the cure and post-cure processing of the getter at elevated temperatures.

To test this hypothesis, we made several 0.010" thick film samples of GE 615 with varying ratios of resin to cure agent. The manufacturer's recommended ratio of resin to silane curing agent is 90/10. We prepared unfilled samples of silicone using this ratio as well as samples using ratios of 93/7, 94/6 and 95/5. After curing for 16 hours at 100°C, the infrared absorbance peak areas of the residual silane groups were measured. The samples were then postcured for 24 hours at 170°C and then again for 24 hours at 200°C, with IR absorbance measurements made after each treatment. The results are shown in Table 12 below:

Table 12

Si-H GROUP IR ABSORBANCE PEAK AREA IN ARBITRARY UNITS for GE 615 RTV SILICONE

Treatment	Resin/Cure Agent Ratio			
	90/10	93/7	94/6	95/5
16 hrs @ 100°C in air oven	31.3	13.6	12.0	7.8
Add 24 hrs @ 170°C in vacuum oven	12.3	4.99	2.93	1.33
Add 24 hrs @ 200°C in vacuum oven	6.11	2.00	1.83	1.11

An examination of the data in the above table reveals two significant facts. First, a lowering of the silane cure agent does indeed reduce the residual silane group concentration left in the cured silicone polymer. Second, thermal treatment also reduces the residual silane concentration. Both these effects should therefore reduce the potential hydrogen evolution by the silicone polymer, allowing the getter to retain a capacity closer to the theoretical value after processing. Subsequent capacity testing indicated that the lower cure agent formulations did indeed have higher capacities, with the 95/5 mix yielding essentially the theoretical value of approximately 27 cc-atm/gram.

We also performed an aging test at 150°C of samples of 90/10 and 95/5 mix ratios, wherein the evolved hydrogen versus time was measured. The samples were cured for 16 hours at 100°C and postcured for 24 hours at 150°C. Two samples of each mix were aged in stainless steel sample vessels from which gas samples could be withdrawn for analysis. Each sample vessel was evacuated and backfilled with dry nitrogen to 1100 torr prior to the start of the aging test. Two blank steel vessels were also included in the test

so that the results could be corrected for the small amount of hydrogen that would be evolved from the steel itself. The results are shown in the Table 13 below:

Table 13

Time (hrs)	Hydrogen Concentration (%)					
	90/10 14.163g	90/10 10.974g	95/5 11.963g	95/5 15.618g	blank 0g	blank 0g
92	1.65	1.24	ND	ND	ND	ND
428	2.04	1.55	0.032	0.027	0.009	0.010
840	2.28	1.82	0.028	0.033	0.021	0.018
1464	2.49	1.94	0.035	0.043	0.035	0.041
2808	3.03	2.40	0.081	0.111	0.095	0.095

The results in Table 13 confirm that the hydrogen evolution from the silicone polymer may be reduced by lowering the amount of silane cure agent used to prepare the mix. The hydrogen values from the 95/5 ratio mix differ little if any from that seen in the empty vessels. Based on the results of these tests and the tests of hydrogen capacity, all getter samples for all other examples herein, except for examples 11-13, were prepared using the 95/5 mix ratio and the best results for GE 615 is about that ratio. Examples 11-13 used a 90/10 mix ratio. For other binders, similar tests would need to be performed to find the preferred mix of resin and hardener to minimize unreacted binder.

CLAIMS

What is claimed is:

1. A hydrogen gas absorber comprising effective amounts of oxides of platinum group metals to remove hydrogen from a particular environment, effective amounts of a desiccant to absorb water produced from the reaction of hydrogen with said oxides, and effective amounts of a gas permeable binder to satisfactorily hold said oxides and said desiccant in a matrix formed by said binder.

2. An absorber as claimed in claim 1 wherein the amounts of said oxides are from about 10 to about 30 percent by weight and the amounts of said desiccant are from about 5 to about and 35 percent by weight and the amounts of said binder are from about 85 to about 35 percent by weight.

3. An absorber as claimed in claim 2 further comprising effective amounts of a radio frequency absorbing substance to absorb unwanted radio frequency waves.

4. An absorber as claimed in claim 2 wherein said oxides are produced by a salt precipitation technique and contains less than about 200 parts per million by weight of chlorine, wherein said desiccant is a molecular sieve desiccant, and wherein said binder is a room temperature vulcanized silicone.

5. An absorber as claimed in claim 4 further comprising effective amounts of a platinum group metal to accelerate reaction of hydrogen gas with unreacted oxygen.

6. A hydrogen gas absorber comprising from about 10 to about 30 percent by weight palladium monoxide, from about 5 to about and 35 percent by weight of a molecular sieve desiccant, and from about 85 to about 35 percent by weight of a gas permeable binder, said palladium monoxide and said desiccant held in a matrix formed by said binder.

7. An absorber as claimed in claim 6 wherein said oxides are produced by a salt precipitation technique and contains less than about 200 parts per million by weight of chlorine, wherein said desiccant is a molecular sieve desiccant, wherein said binder is a room temperature vulcanized silicone consisting of a resin reacted with a cure

agent, and wherein the ratio of said cure agent to said resin is such that the amount of said cure agent that is unreacted residual is minimized.

8. An absorber as claimed in claim 7 further comprising effective amounts of a radio frequency absorbing substance to absorb unwanted radio frequency waves.

9. An absorber as claimed in claim 7 further comprising effective amounts of a platinum group metal to accelerate reaction of hydrogen with unreacted oxygen.

10. A hydrogen gas absorber hermetically-sealed electronics enclosure system comprising: a hermetically sealed enclosure; semiconductor electronic components contained within said enclosure; and a hydrogen gas absorber contained within said enclosure comprising from about 10 to about 30 percent by weight of oxides of platinum group metals, from about 5 to about and 35 percent by weight of a desiccant, and from about 85 to about 35 percent by weight of a gas permeable binder to hold said oxides and said desiccant in a matrix formed by said binder.

11. A method of manufacturing a hydrogen absorber system comprising the step of: mixing effective amounts of oxides of platinum group metals to remove hydrogen from a particular environment, effective amounts of a desiccant to absorb water produced from the reaction of hydrogen with said oxides, and effective amounts of a gas permeable binder to satisfactorily hold said oxides and said desiccant in a matrix formed by said binder.

12. The method as claimed in claim 11 wherein the amounts of said oxide of platinum group metals are from about 10 to about 30 percent by weight and the amounts of said desiccant are from about 5 to about and 35 percent by weight and the amount of said gas permeable binder is from about 85 to about 35 percent by weight.

13. A method as claimed in claim 11 wherein said oxides are produced by a salt precipitation technique and contains less than about 200 parts per million by weight of chlorine, wherein said desiccant is a molecular sieve desiccant, and wherein said binder is a room temperature vulcanized silicone consisting of a resin reacted with a cure agent and wherein the ratio of said cure agent to said resin is such that the amount of said cure agent that is unreacted residual is minimized.

14. A method of gettering hydrogen from a hermetically sealed vessel comprising the steps of: placing effective amounts of oxides of platinum group metals, effective amounts of a desiccant, and effective amounts of a gas permeable binder to satisfactorily perform hydrogen absorption in a hermetically sealed vessel.

15. The method as claimed in claim 14 wherein the amounts of said oxide of platinum group metals are from about 10 to about 30 percent by weight and the amounts of said desiccant are from about 5 to about and 35 percent by weight and the amount of said gas permeable binder is from about 85 to about 35 percent by weight.

INTERNATIONAL SEARCH REPORT

Inten nal Application No
PCT/US 96/15540

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C01B3/58 H01L23/26 C08K3/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C01B H01L C08K H01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 496 711 A (SAES GETTERS SPA) 29 July 1992 see page 2, line 49 - page 3, line 24 see claims	1,2,4-7, 9-15
Y	WO 86 03056 A (MESSERSCHMITT BOELKOW BLOHM) 22 May 1986 see page 3, line 26 - line 29 see claims 1,2; example 1	1,2,4-7, 9-15
X	GB 1 339 524 A (HUGHES AIRCRAFT CO) 5 December 1973 see page 1, line 24 - page 2, line 58 see page 3, line 40 - line 52	1,2,6, 10,11
A	US 4 405 487 A (HARRAH LARRY A ET AL) 20 September 1983 see claims	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1997	07.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Van der Poel, W

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INTERNATIONAL SEARCH REPORT

Inten nal Applicatoo No
PCT/US 96/15540

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 96/15540

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